UNIVERSITY OF OSLO

FACULTY OF MEDICINE

The pathogenic role of tumor necrosis factor superfamily ligands in atherogenesis and plaque destabilization

Wiggo Johan Sandberg

Research Institute for Internal Medicine

Section of Clinical Immunology and Infectious Diseases

Department of Cardiology

RIKSHOSPITALET UNIVERSITY HOSPITAL

Faculty of Medicine

UNIVERSITY OF OSLO

2008

The Norwegian Council on Cardiovascular Diseases

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 707

ISBN 978-82-8072-772-5

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2008.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Unipub AS is owned by
The University Foundation for Student Life (SiO)

TABLE OF CONTENTS

1. Acknowledgements

This thesis is based on studies performed at the Research Institute of Internal Medicine, Rikshospitalet Medical Centre, University of Oslo, during the years 2004-2008. In this period I was a research fellow financially supported by grants from the Norwegian Council on Cardivascular Diseases and Medinnova AS, for which I am very grateful.

I foremost wish to express my most sincere gratitude to my principal supervisor Professor Pål Aukrust, who initiated the project and has been uniquely supportive throughout my studies. I am truly privileged to have experienced his enthusiasm, prospered on his creativity and learned from his constructive approach to research. His knowledge in the fields of research and immunology has deeply impressed me, and I am thankful for his daily presence and availability for questions and discussions. Most of all I appreciate his friendly nature, who positively affects everyone around him.

I am also grateful to my co-supervisor dr.philos Arne Yndestad, an excellent researcher with a reliable experience I greatly have benefited from. As a living lexicon in both cytokines and jazz, Arne has been an invaluable colleague both professionally and socially. I am also deeply indebted to dr.philos Bente Halvorsen, for being a methodological mentor and for her substantial contribution to my thesis. I am also most grateful to Ellen Lund Sagen and Turid Magrethe Pedersen, for their skillful technical assistance and friendliness.

Next I want to thank my closest colleagues, my room mate Camilla Smith for many good laughs and for being an outstanding teammate, Thor Ueland for technical wizardry, Erik Øie for great and reliable support, Morten Mattingsdal for his complex microarray analysis, Ernst Kristian Rødland, Jan Kristian Damås, Christen Dahl, Unni Bredland, Linn Landrø, Børre Fevang, Tove Lekva, Kari Otterdal, Annika Michelsen, Kirsten Holven, Hanne Scholtz, Bodil Lunden, Azita Rashidi, Cecilia Guevara, Vigdis Bjerkeli, Maureen Raw and all my other co-workers, for down to earth attitude and loads of fun. The efforts of all co-authors are also greatly acknowledged.

I also want to thank Professor Stig Frøland, Section of Clinical Immunology and infectious Diseases, for contributing with his great knowledge and critical approach to research and manuscript preparation. Thanks also go to Professor Frank Brostad and Professor Nils Olav Solum, for providing me with excellent working facilities at the Research Institute for Internal Medicine and for sharing their great knowledge in the field of platelet biology. Thanks are also due to Professor Lars Gullestad, Dr. Arne Andreassen and Professor John Kjekshus, Department of Cardiology, for a highly productive collaboration and for sharing their expertise in cardiology.

Thanks also go to our collaboration partners in Sweden, represented by Professor Gøran K Hansson, Center of Molecular Medicine, Karolinska Institute, Stockholm, Sweden, for sharing their expertise.

The patients who in spite of their difficult situation willingly participated in our studies also truly deserve honorable mention. Without their contribution this work would have been impossible, and I am deeply grateful.

My warmest thoughts go to my friends and family, thanks to all my sisters for being supportive, Silje and Steinar for their genuine interest in my field, and to my parents for their encouragement. Above all, I am grateful to Janne for her great support and love. You are all tremendously important to me.

2. Abbreviations

ACS	Acute coronary syndromes
apo	Apolipoprotein
APRIL	A proliferation inducing ligand
CAD	Coronary artery disease
CD40L	CD40 ligand
CRP	C reactive protein
CSF	Colony stimulating factor
HDL	High density lipoprotein
HVEM	Herpes virus entry mediator
IFN	Interferon
IL	Interleukin
LDL	Low density lipoprotein
LIGHT	Lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D (gD) for HVEM, a receptor expressed by T lymphocytes
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant peptide
MAPK	Mitogen activated protein kinase
MI	Mycardial infarction
MMP	Matrix metalloproteinase
NFκB	Nuclear factor-κB
NYHA	New York heart association
OPG	Osteoprotegerin
oxLDL	oxidized LDL
PCR	Polymerase chain reaction
PAR	Proteinase activated receptor
PCI	Percutaneous coronary intervention
RANKL	Receptor activator of NFκB ligand
ROS	Reactive oxygen species
SAP	Stable angina pectoris
SR-A	Scavenger receptor-A
SMC	Smooth muscle cells
STEMI	ST-segment elevation MI
TNF	Tumor necrosis factor
TNFRSF	TNF receptor superfamily
UAP	Unstable angina pectoris

3. List of papers

Paper I:
Wiggo J. Sandberg, Arne Yndestad, Erik Øie, MD, Camilla Smith, Thor Ueland, Olga Ovchinnikova, Anna-Karin L. Robertson, Fredrik Müller, MD, Anne G. Semb, MD, Hanne Scholz, Arne K. Andreassen, Lars Gullestad, Jan Kristian Damås, Stig S. Frøland, MD, Göran K. Hansson, Bente Halvorsen, Pål Aukrust. *Enhanced T-cell expression of RANK ligand in acute coronary syndrome – possible role in plaque progression*
Arteriosclerosis Thrombosis and Vascular Biology 2006 Apr;26(4):857-63

Paper II:
Hanne Scholz, Wiggo Sandberg, Jan Kristian Damås, Camilla Smith, Arne K. Andreassen, Lars Gullestad, Stig S. Frøland, Arne Yndestad, Pål Aukrust, Bente Halvorsen. *Enhanced plasma levels of LIGHT in Unstable Angina: Possible pathogenic role in foam cell formation and thrombosis*
Circulation 2005 Oct 4;112(14):2121-9.

Paper III:
Wiggo J. Sandberg, Bente Halvorsen, Arne Yndestad, Camilla Smith, Kari Otterdal, Frank R. Brosstad, Stig S. Frøland, Peder S. Olofsson, Jan K. Damås, Lars Gullestad, Göran K. Hansson, Erik Øie, Pål Aukrust. *Inflammatory interaction between LIGHT and Proteinase-activated receptor-2 in endothelial cells – potential role in atherogenesis*
Submitted. (Revised version accepted in Circulation Research)

Paper IV:
Wiggo J. Sandberg, Kari Otterdal, Lars Gullestad, MD, Bente Halvorsen, Asgrimur Ragnarsson, Stig S. Frøland, Jan K. Damås, Erik Øie, Pål Aukrust, Gøran K. Hansson, Arne Yndestad. *Platelets release the tumor necrosis factor superfamily ligand APRIL (TNFSF13) upon activation – increased expression of APRIL in coronary and carotid atherosclerosis*
Submitted.

4. Introduction

4.1 General background

Atherosclerotic disorders are the underlying cause of most cases of myocardial infarction, stroke and gangrene, are the most common lethal disease in Western societies and are expected to be the number one killer globally in 2020.[1] Despite better understanding of the pathobiology, knowledge of risk factors and improved treatment strategies, CAD and other atherosclerotic disorders, still remains a progressive disease with high morbidity.

4.1.1 Epidemiology and risk factors

Back only to the mid-nineties, optimistic views held that CAD would be eliminated by the end of the 20th century due to treatment of hypercholesterolemia and hypertension. But rapidly increasing prevalence in developing countries[2] and eastern Europe as well as rising incidence of obesity and diabetes in the western world have defied this prediction and challenge us to consider new strategies for prediction, prevention and treatment.[3] Currently, cardiovascular disease cause 38 percent of all deaths in North America and are the most common cause of death in European men under 65.[4]

About one of four deaths in Norway is caused by CAD. The incidence is higher in men, and rises with age in both sexes. Above the age of 60 the difference between sexes declines, but never disappears. Incidence of symptomatic CAD in Norway is described in the population-based "Tromsø-study" which included 27,000 persons above 25 years. Here, 4.1% of the men and 1.6% of the women reported that they had had a myocardial infarction (MI). Further, 2.6% of the participants reported that they were suffering from angina pectoris.

The most established risk factor of CAD is hypercholesterolemia (elevated low-density lipoprotein [LDL] cholesterol).[5] However, atherosclerotic progression vary considerably at any given plasma cholesterol level, suggesting that other risk factors may be involved.[6] Other risk factors, predisposing factors and factors that are associated with cardiovascular risk are presented in Table 1.

Table 1. Proven and putative risk factors of cardiovascular diseases[#]

Risk factors that are casually linked:
1. Tobacco consumption
2. Elevated LDL
3. Low HDL
4. High blood pressure
5. Elevated glucose
6. Physical inactivity*
7. Obesity*
8. Diet*
Risk factors that show associations:
1. Elevated prothrombotic factors
2. Markers of infection and inflammation
3. Elevated homocysteine
4. Elevated lipoprotein (A)
5. Low socioeconomic status*
6. Psychological factors and breakdown of social structures*

*Predisposing risk factors: A predisposing risk factor is presumed to work, at least partly, through an impact on other risk factors that act directly. It is likely that some of the predisposing factors also have direct effects.
[#]Modified from Yusuf[5]

4.1.2 Clinical features of atherosclerosis

The clinical manifestations of CAD roughly divide in two entities: chronic ischemic CAD, which often manifests as stable angina but may also be asymptomatic, and acute coronary syndromes (ACS), which include unstable angina pectoris (UAP), myocardial infarction (MI) and sudden cardiac death. Atherosclerotic coronary artery disease manifests in the arteries as an asymmetric focal thickening of the intima by accumulation of lipid droplets and immune cells, typically evolving into a stenotic lesion that encroach luminal space and restrict blood flow. Thus, particularly under situations of increased cardiac demand, ischemia occurs, leading to symptoms of stable angina pectoris.[7,8] These are typically chest pain and/or dyspnea induced by physical exertion or psychological stress. Grading of the symptoms of angina pectoris may be done according to consensus systems such as the New York Heart Association (NYHA) classification and the Canadian Cardiovascular Society classification which both classify severity from I to IV.[9] The diagnosis of stable angina is based on typical clinical history, exercise

electrocardiography (ECG), nuclear techniques for evaluation of myocardial perfusion, and often coronary angiography revealing significant stenosis in the coronary vessels. In contrast to the progressive stenosis of stable angina, unstable angina and acute myocardial infarction seem to be caused by a sudden physical disruption of the atherosclerotic plaque, triggering subsequent thrombus formation and vascular obstruction. Unstable angina is clinically defined as chest pain at rest within the past 48 hours; a classification is presented by Braunwald.[10,11] The diagnostic criteria is based on biochemical markers of myocardial injury, particularly the cardiac troponins I and T but also creatine kinase (CK)-MB mass. The patient further may present with or without electrocardiographic changes, i.e., transient ST-segment depression or T-wave inversion, and with or without elevated myocardial markers as troponin T or troponin I. The presence or absence of these characteristics (ECG changes and/or elevated troponins or no changes) put the patient into a high-risk or low-risk state for developing acute MI or death within 30 days.[11]

In 2000, The Joint European Society of Cardiology/American College of Cardiology (ESC/ACC) Committee for the redefinition of myocardial infarction[12] suggested that any amount of necrosis resulting from ischemia should be diagnosed as a MI. As a consequence of this new definition, patients who would previously have been considered to have unstable angina are being diagnosed with a MI. Accordingly, patients with MI are divided into ST-segment elevation MI (STEMI) and non-STEMI. In a recent study, STEMI patients were more likely to receive fibrinolysis or undergo percutaneous coronary intervention (PCI), but less likely to have bypass surgery. At discharge, patients with STEMI received more aggressive secondary prevention therapies than those with NSTEMI, which was not supported by differences in disease severity.

4.1.3 Current treatment strategies

Treatment of cardiovascular disease has improved during the last 20 years, both with respect to revascularization with bypass grafting or PCI, and evidence-based medical therapy, which in principle rests on three cornerstones: anti-platelet, anti-coagulant, and fibrinolytic therapy; vaso-relaxing treatment by beta adrenergic (β-) blockers and nitrates;

and cholesterol-lowering therapy. Anti-platelet therapy is the basic treatment strategy, and aspirin is currently the most used drug, reducing the risk of death and MI in both stable and unstable angina.[13-16] The thienopyridines ticlodipine and clopidogrel and intravenous glycoprotein IIb/IIIa inhibitors may be well as effective in preventing coronary events as aspirin, recommended if aspirin-intolerance and as a bridge to and after acute PCI, respectively.[17, 18] Moreover, the coagulation inhibitor heparin adds to the anti-coagulation treatment strategy of unstable angina.[19] Thrombolytic or fibrinolytic therapy is also frequently used, in particular where facilities for PCI are not available. β-blockers give symptomatic relief in both stable and unstable angina, and risk reduction is evident in patients with previous MI or reduced left ventricular function. However, data from randomized trials supporting risk reduction in angina are more limited.[20] Nitrates and selected calcium antagonists are well-established as symptom relievers, but have no risk-reducing effects. Finally, cholesterol-lowering therapy with statins is highly effective in reducing the risk of major events in all groups of CAD, both chronic and acute.[21-26] However, despite state-of-the art treatment, CAD is a progressive disease with high morbidity and mortality, suggesting that pathogen mechanisms remain active and unmodified by current treatment modalities. In our opinion, persistent immune activation may represent such mechanism.

4.1.4 The atherosclerotic process

The normal intimal layer of an artery is covered on the luminal surface of a monolayer of endothelial cells that is critical for vascular homeostasis. Below intima is the media layer, containing mainly smooth muscle cells (SMCs), and finally the outer adventitia layer, containing connective tissue, collagen and elastic fibers. Several mechanisms have been proposed to explain the initiating events in atherosclerosis, and three main hypotheses have emerged; the response-to-injury, the response-to-retention and the oxidative modification hypothesis.[27-29] These hypotheses are now considered to present the main contributory factors in initiation of atherosclerosis, not mutually exclusive but compatible with each other, explaining how endothelial cells become activated by risk factors such as hypercholesterolemia, tobacco smoking, inflammation and disturbed hemodynamic flow. The activated endothelium has increased permeability and invasion of T cells and

monocytes as well as accumulation of lipoproteins in the intima are hallmark characteristics of atherosclerosis.[30] Here, according to the oxidation hypothesis, LDL cholesterol is tightly adhered to proteoglycans, undergoing progressive oxidation in the microenvironment which is relatively sequestered from the plasma antioxidants.[6] Simultaneously, intimal monocytes proliferate and differentiate to macrophages which perform rapid uptake of oxidized LDL (oxLDL) particles, subsequently transforming the cells into lipid-laden macrophages or so called foam-cells. These are the dominant cell type in the early atherosclerotic plaque, or "fatty streak", which is prevalent in younger individuals and cause no symptoms. This nascent lesion can either disappear with time or evolve to stenotic atheromas. Further plaque progression consists of recruitment and mitosis of leukocytes as well as migration and proliferation of vascular SMC and continued accumulation of LDL, creating the fibro-fatty plaque typically characterized as a foam-cell-surrounded, lipid-rich, necrotic core, surrounded by a cap of SMC and collagen-rich matrix.[31] The plaque may be "silent" or lead to ischemia with symptoms of stable angina pectoris. However, ACS, i.e., unstable angina and acute MI, seem to be caused by plaque disruption, rather than complete stenosis; rupture of the fibrous cap promote thrombus formation that either may lead to partly or totally occlusion of the vessel lumen and acute disease. Resorption of thrombi often causes healing responses that cause the fibro-fatty plaque to evolve into a fibrous and often calcified plaque with significant stenosis. [30]

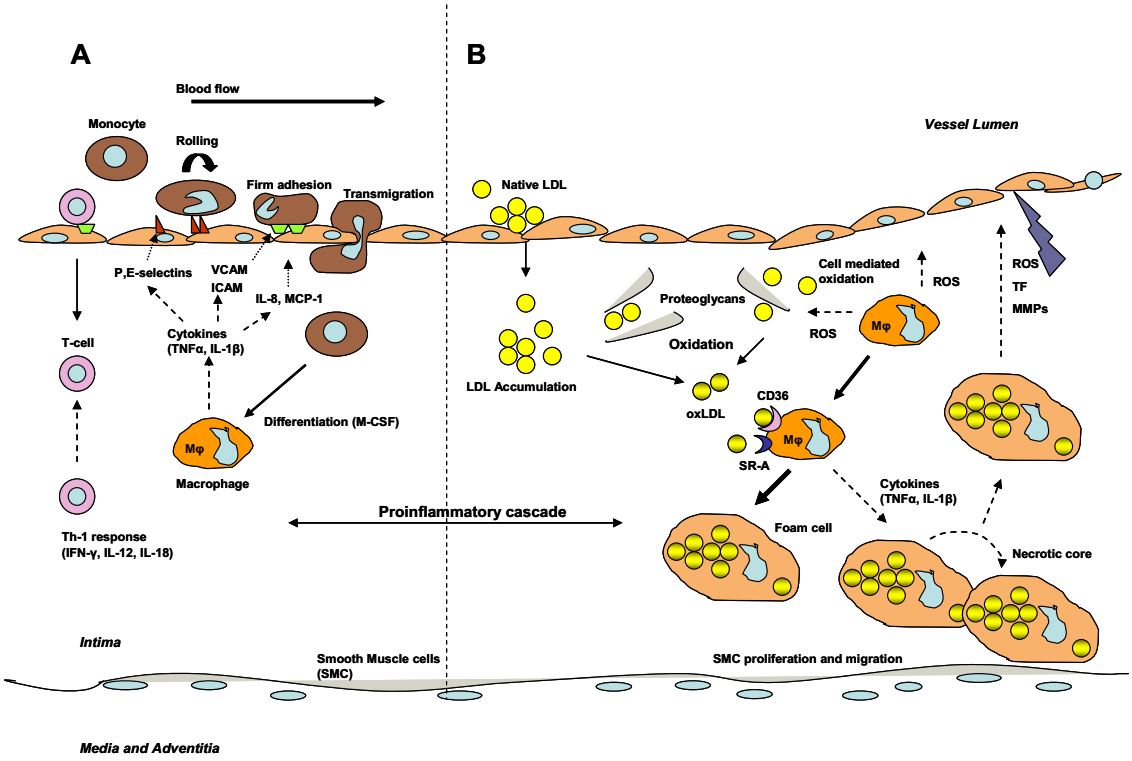

Figure 1. Atherogenesis – interaction between lipids and inflammation

The atherosclerotic process in the artery wall is initiated by interaction of endothelial dysfunction (panel A) and retention, oxidative modification and scavenging of LDL in the intima (panel B). Endothelial dysfunction is characterized by increased endothelial permeability and increased expression of adhesion molecules, chemotactic proteins and growth factors causing adhesion, rolling and migration of monocytes and T-cells into the intima. After entering the intima, monocytes differentiate into macrophages that release reactive oxygen species (ROS) and several cytokines leading to enhanced expression of adhesion molecules and subsequent monocyte migration. This promotes the pro-inflammatory cascade with activation of T cells secreting mainly Th-1 cytokines, activating macrophages and other intima cells leading to inflammation. Endothelial dysfunction also leads to increased passive transport of LDL into the subendothelial space, where it interact with and is retained by proteoglycans. At limiting levels of antioxidants, the lipids and proteins of LDL undergoes oxidative modifications as a result interaction with ROS and enzymes, forming oxidized LDL (oxLDL) which in turn further activate endothelial cells. Uptake of oxLDL by scavenger receptors on macrophages leads to foam cell formation, which leads to liberation of ROS and inflammatory cytokines, thus promoting further oxidation of LDL and endothelial dysfunction. This process may continue by SMC proliferation and migration from the media to the intima, as well as collagen deposition, creating a fibrous cap surrounding a lipid-rich necrotic core and dead or dying SMCs and macrophages. This constitutes the atherosclerotic plaque which leads to focal thickening of the media and restriction of blood flow, thus angina pectoris. Continuous monocyte influx, SMC proliferation and pro-inflammatory positive feedback, give rise to the more advanced atherosclerotic lesions. In acute coronary syndromes, matrix metalloproteinases (MMPs) digest collagen and cause thinning of the fibrous cap sequestering the lipid-laden core from the blood-flow, eventually resulting in plaque rupture and thrombus formation in the vessel.

4.2 The role of inflammation in atherosclerosis and plaque destabilization

4.2.1 Inflammatory mediators – cytokines

Cytokines are small proteins produced and secreted not only by immune cells but also by most cells in the organism, and mediate intercellular communication. They regulate fundamental biological processes via cell surface receptors, in autocrine, paracrine and sometimes endocrine manners. More than 100 cytokines are reported, conventionally classified into one of five families: interleukins (IL), interferons (IFN), colony stimulating factors (CSF), chemokines (chemotactic cytokines) and the tumor necrosis factor (TNF) superfamily, which will be discussed in detail below. Cytokines are of particular importance as regulators of the immune system. Due to considerable overlap between the families classified above, a subdivision is often preferred, such as dividing the cytokines with pro-inflammatory abilities (classically TNFα, monocyte chemoattractant peptide (MCP)-1 IL-1, IL-6, IL-8 IL-12, IL-18 and INFγ), from those with largely anti-inflammatory abilities (including IL-4, IL-10, IL-13 and transforming growth factor [TGF]-β). However, even this definition may be simplified. Thus, while IL-4 and IL-13 hold anti-inflammatory properties by their ability to down-regulate inflammatory T helper cell type I (Th1) responses, they also are important pathogenic mediators in some inflammatory pulmonary disorders such as asthma.[32-35] Moreover, even if TGFβ are regarded as a potent anti-inflammatory cytokine, it also has monocyte/macrophage chemotactic properties with potential inflammatory consequences. In fact, even the classical anti-inflammatory cytokine IL-10 may, at least through long-time exposure, induce inflammatory effects through its ability to promote B cell activation and antibody production.

Cytokines share a number of specific features: *i)* They show pleiotropic activities; a cytokine can trigger several different cellular responses depending on cell type, timing and context. *ii)* They act synergistically; the association of two different cytokines (for example IL-8 and MCP-1) markedly amplifies their activity. *iii)* They act in an autocrine,

paracrine and juxtacrine manner; cytokines can stimulate the cells that produce them, adjacent cells, or through direct cell-cell interaction. This local mode of action sets cytokines apart from classical hormones. However, under certain circumstances characterized by severe inflammation, endocrine functions may also occur *iv)* They commonly share receptor subunits, and have to a large degree overlapping functions. *v)* Unique is also their tight regulation, separating them from growth factors, which tend to be more constitutively expressed.[36]

4.2.2 Inflammatory diseases - too much of a good thing

While inflammatory cytokines play an important protective role the immune response against foreign pathogens and cancer cells, excessive tissue production can mediate detrimental effects.[37] Thus, enhanced production of inflammatory cytokines has been implicated in the pathogenesis of both acute (e.g., septic shock and cerebral malaria) and chronic (e.g., inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis and human immunodeficiency virus [HIV] infection) conditions.[38, 39] While anti-inflammatory type cytokines such as IL-10 ideally would limit the deleterious effects of inflammatory cytokines, prolonged IL-10 excess may promote progression of autoimmune diseases by enhancing B cell activity and autoantibody production, or immunodeficiency by deactivation of monocytes and macrophages.[40, 41] Further, down-regulation of TNF-α in septic shock seems reasonable from an experimental point of view, since too much TNF-α itself defines septic shock, but anti-TNF treatment was associated with increased mortality potentially due to “too much down-regulation” of this cytokine.[39] Based on promising results in animal models, clinical trials using IL-10 against inflammatory bowel disease and rheumatoid arthritis have been conducted, but shown opposing results.[42, 43] Thus, the balance between beneficial and harmful effects of the same cytokine is of major importance from a therapeutical point of view.

4.2.3 Atherosclerosis – an inflammatory disease

Inflammation plays pivotal roles in all stages of atherosclerosis. The initial onset of atherosclerotic lesion development is related to blood flow disturbances, occurring at branch orifices, bifurcations and inner curvatures. At these sites, low or oscillatory blood flow induce endothelial cells, through shear stress responsive transcription factors, to

express adhesion molecules, chemokines and growth factors.[44] The adhesion molecules, such as selectins (e.g., E-selectin and P-selectin), and members of the immunoglobulin superfamily (e.g., intercellular adhesion molecule-1 [ICAM] and vascular adhesion molecule-1 [VCAM]), which act as receptors for glycoconjugates and intergrins expressed on monocytes and T cells, and cause rolling and firm adhesion of leukocytes onto endothelial cells with subsequent migration of leucocytes into the intima.[45] Flow disturbances may also directly induce MCP-1 expression in endothelial cells,[44] which amplifies the recruitment and invasion of monocytes to the vascular wall.

Once resident in the intima, monocytes differentiate into macrophages in response to macrophage colony stimulating factor (M-CSF)-stimulation, increase expression of scavenger receptors such as CD36 and scavenger receptor-A (SR-A) with subsequent enhanced uptake of modified lipoproteins such as oxLDL. Macrophages have a potential immunomodulatory capasity[46] and secrete a wide range of cytokines, such as MCP-1 and IL-8, both potent chemoattractant chemokines that recruits leucocytes, as well as IL-12, a potent activator of T cells. The roles of T cells in the plaque are dual, due to T cell subsets with different effect on atherogenesis.[47] Thus, while T cells are thougt to promote atherogenesis, there are also T cell subsets with potential anti-atherogenic properties, such as regulatory T cells (T_{reg}, $CD4^+CD25^+$), specialized in suppression of pathogenic Th1 and Th2 responses against self and foreign antigens. Being a major source of the anti-inflammatory cytokines IL-10 and TGF-β, T_{reg} have been demonstrated as powerful inhibitors of atherosclerosis in several mouse models.[48-50] In addition to cytokine-dependent suppression of immune responses, T_{reg} may also suppress pathogenic T cells through cell-cell contact-dependent mechanisms, particularly through engagement of cytotoxic T lymphocyte antigen (CTLA)-4 expressed on T_{reg} with CD80/CD86 molecules expressed on effector T cells, or through interaction of CTLA-4 with CD80/CD86 on antigen presenting cells, leading to up-regulation of indoleamine dioxygenase and indirect down-regulation of effector T cell responses through tryptophan catabolism.[51] Moreover, Th2 cells have been associated with increased production of protective anti-oxLDL antibodies, which are shown to reduce lesion size. [4, 52, 53] However, as discussed above, IL-4 may also have inflammatory properties and deficiency in IL-4 was recently shown to be associated with a decrease in atherosclerotic lesion formation, particularly at

the advanced stages of lesion progression.[54] While the role of Th2 cells is debated, there is solid evidence from several independent groups that the Th1 subset is a particular proatherogenic subset within the $CD4^+$ T cell population. First, a number of studies have co-localized $CD4^+$ T cells and IFNγ within human and mouse atherosclerotic lesions, suggesting predominance of Th1 cells in atherogenesis. [55-57] More recently, high levels of IL-12 and IL-18 mRNA and protein have also been detected in atherosclerotic plaques further suggesting a Th1 profile in these lesions.[58] Second, a direct role in atherogenesis has been defined in atherosclerotic-susceptible mice that are deficient in either IFNγ receptors [59] or the cytokine itself.[60] Conversely, injection of IFNγ or the IFNγ–releasing factors IL-12 and IL-18 enhances the extent of disease in $apoE^{-/-}$ mice.[61]

However, the production of cytokines within an atherosclerotic plaque is not restricted to T cells and macrophages. In fact, all cell types present in the atherosclerotic plaque are potential contributors pro-inflammatory cytokines.[36] Thus, endothelial cells, mast cells and even SMC has been shown to produce numerous cytokines and chemokines, promoting leukocyte migration and inflammation.

4.2.4 Plaque-related inflammation: Promotion of matrix degradation and thrombus formation

Within the atherosclerotic plaque, inflammation may have several pathogenic consequences, and its effect on matrix regulation and thrombus formation may be of particular importance. Thus, SMC respond to IL-1 and indirectly, to IFNγ with proliferation and migration to the site of the atherosclerotic plaque.[62, 63] In the growing mass of extracellular lipid, intimal SMC migrate and deposit collagen and other extracellular matrix proteins, forming a fibrous plaque that protrudes in to the arterial lumen, eventually leading to formation of the fibrous cap surrounding the lipid-rich necrotic core that constitutes the atheroma. Moreover, the inflammatory cytokines produced during atherosclerosis significantly modulate the extracellular matrix composition, by affecting the expression of matrix metalloproteinases (MMPs) and their inhibitors tissue inhibitors of MMP (TIMP). Matrix degrading MMP production can be increased by oxidized lipids, reactive oxygen species, heat shock proteins, inflammatory cytokines and hemodynamic stress, all components existing in the plaque. Thus,

inflammation may lead to a shift of the plaque proteome toward matrix degradation, affecting plaque stability by thinning of the fibrous cap, leaving the plaque vulnerable to rupture. Cytokines also may promote thrombus formation within the lesion by causing enhanced expression of tissue factor (TF), a potent procoagulant, in plaque cells.[64] Moreover, cytokines can up-regulate plasminogen activator inhibitor (PAI)-1 and down-regulate thrombomodulin, a potent endogenous anti-coagulant, further contributing to a prothrombotic state within the lesion. Hence, if the weakened cap ruptures, coagulation factors in blood gain excess of thrombogenic TF, causing thrombosis as described above. Moreover, due to cytokine-mediated effects on PAI-1 and thrombomodulin, the fibrinolytic and anti-thrombotic potential within the lesion will be decrased, further promoting thrombus formation at the site of plaque rupture.

4.2.5 Immune-mediated plaque destabilization

Two major types of physical disruption of the atherosclerotic plaque may occur.[65] Superficial erosion of the endothelial monolayer uncover subendothelial collagen and von Willebrand (vW) factor, promoting platelet adhesion and activation,[66] thus making a nidus of a platelet thrombus formation with subsequent MI, counting for one-quarter of fatal coronary thromboses. The most common mechanism of plaque disruption involves rupture of the fibrous cap.[30, 65] The thrombogenic, lipid-rich core of the plaque is normally sequestered from the blood-flow by the fibrous cap, but upon fissure formation, usually at the shoulders of the plaque, exposure and activation of the coagulation cascade in the blood vessel occurs, causing approximately three-quarters of all MI. Inflammation is instrumental in both of these mechanisms. First, endothelial erosion is linked to the proximity of highly activated subendothelial macrophages that may cause endothelial cell death by apoptosis and also weaken the endothelial intercellular integrity by producing MMPs. Second, a high content of SMC are thought to make the plaque less prone to rupture, and cytokine-induced apoptosis of these cells may also weaken the overall integrity of the plaque. Typical features of the vulnerable plaque are: a large lipid core occupying at least 50% of the plaque volume, a high density of macrophages, a high TF content, and low content of SMC and collagen in the fibrous cap.[67] Although several cell types may contribute to these features of plaques at future risk, macrophage activation

seems to be of particular importance. Thus, the site of rupture is characterized by enhanced inflammation and often occurs in the macrophage-rich shoulder region.[68] Notably, plaque rupture is also influenced by external factors such as turbulence in passing blood, mechanical stress etc.[65] Further, recent studies have shown that in addition to plaque size, the orientation of remodeling plays a role on vulnerability. Positive remodeling is characterized by "outward expansion", that is, the plaque expands into the vessel wall, whereas negative remodeling encroach lumen. Positive remodeling is associated with unstable angina, whereas negative remodeling is more common in stable angina.[69-71] Accordingly, several studies have suggested that in 60-70% of patients with ACS, the culprit site of the acute event had less than 70% (often under 50%) of vessel diameter narrowing.[72] Thus, plaques producing non-flow-limiting and less than severe stenosis, accounts for more cases of ACS events than the severe stenotic plaques producing symptoms of stable angina. Moreover, recurrent coronary events, frequently occurring in ACS, are unrelated to the culprit lesion in almost half of the cases,[73] supporting the view of widespread diseased coronary vessels with multiple vulnerable plaques, reflecting widespread coronary inflammation.[74, 75] It is also important to have in mind that, despite the body of evidence of a link from plaque vulnerability, rupture and ACS, many plaque ruptures do neither cause any occlusion of the vessel nor clinical symptoms.[65]

4.2.6 Triggers of inflammation in atherosclerosis

Chronic infections caused by for example *Chlamydia pneumonia* have been suggested to be involved in atherogenesis, and persistent stimulation by microbial antigens might well lead to enhanced inflammation within an atherosclerotic plaque. First, chronic extravascular infections (e.g. gingivitis and bronchitis) may augment production of inflammatory cytokines that may accelerate the evolution of remote atherosclerotic lesions.[76-78] Second, many human plaques show sign of infection by microbial agents such as *Chlamydia pneumonia,*[79-81] and indeed, infection with *Chlamydia pneumonia* has been found to accelerate atherosclerosis in rabbits on an atherogenic diet.[82, 83] *Chlamydia,* when present in arterial plaques, may release lipopolysaccharide (LPS) and heat-shock protein (Hsp) that can stimulate the production of inflammatory mediators in infiltrating

leukocytes, vascular SMC and endothelial cells.[84] Hsp, besides being molecular chaperons, are immunogenic molecules, and a significant element of the immune response to microorganisms is directed towards Hsp-derived peptides.[85] Interestingly, both microbial antigens and Hsp may induce signaling through toll like receptors (TLRs). Thus, ligation of these receptors, defined as a key component of pathogen-associated molecular pattern recognition machinery, initiates activation of the transcriptional factor nuclear factor (NF)κB resulting in the expression of a wide array of inflammatory genes.[86-88] Interestingly, recent reports have shown that the expression of TLRs, in particular TLR1, TLR2 and TLR4, is markedly increased in human atherosclerotic lesions, and this enhancement occurs preferentially in endothelial cells and macrophages.[89, 90] A recent study reporting an association between a decreased risk of atherosclerosis and TLR4 polymorphism, further supporting a role of TLRs in atherogenesis.[91, 92] Moreover, in this study TRL4 polymorphism and decreased risk of atherosclerosis was not associated with decreased occurrence of bacterial infections, but with an attenuated inflammatory response to such infections. This finding suggests that rather than the microbes themselves, the inflammatory response to these microorganisms seems to be of major importance in the progression of atherosclerosis.

However, the ”inflammation hypothesis” does clearly not depend on the ”infection hypothesis”, and several other factors may lead to cytokine activation and inflammation within the atherosclerotic lesion.[93] For example, modified autoantigens such as oxidized lipoproteins, human derived Hsp and reactive oxygen species (ROS), known to play a pathogenic role in CAD, might through monocyte activation or direct effects on T cells, also lead to increased production of inflammatory cytokines contributing the persistent inflammation within the atherosclerotic lesion.[94] Classical risk factors for atherosclerosis, such as hypertension, hypercholesterolemia, smoking and diabetes mellitus share the ability to induce production of oxygen-free radicals, thus creating oxidative stress which in turn stimulate inflammatory gene expression.[95] Further, oxidative stress mediates pro-inflammatory modifications of lipoproteins, which in turn are major stimulus of inflammation, leading to T cell activation[94] and monocyte differentiation, as described above. Thus, it seems that several factors may contribute to the persistent inflammation within the atherosclerotic lesion, including microbial and

non-microbial antigens as well as antigen-independent mechanisms (e.g., direct T cell activation by IL-7 and IL-15 [96, 97]). Moreover, even traditional risk factors seem to be operating, at least partly, through their ability to enhance inflammatory responses within the lesion, representing a common pathway for a wide range of pro-atherogenic factors.

4.3 The TNF Superfamily

4.3.1 General background

Based on their abilities to kill murine fibrosarcoma cells, the tumor necrosis factors gained their name in 1975, and about 10 years later the two archetypical TNF members were isolated and characterized, namely TNF-α and lymphotoxin β (LTβ). Research during the last three decades have revealed several additional members, and the superfamily now consist of 20 members that signal through 29 different receptors.[98] The TNF receptor superfamily (TNFRSF) are expressed by a wide variety of cells, while the TNF superfamily (TNFSF) ligands are known to be expressed predominately by cells of the immune system, including B cells, T cells, natural killer cells, granulocytes, monocytes and dendritic cells, but also by cardiomyocytes, bone-related cells and endothelial cells.[98] The TNFSF have unique structural attributes that couples them directly to pathways for cell proliferation, differentiation and survival. Within the immune system, their rapid and potent signaling capabilities are crucial in coordinating the proliferation and protective functions of pathogen-reactive cells such as lymphocytes, granulocytes and macrophages.

The TNF superfamily ligands are transmembrane type II proteins, with a C-terminal receptor-binding extracellular domain, that may or may not be shredded by proteinases to yield a soluble form. Most members of the TNFRSF are type I transmembrane proteins, which share a structural hallmark of repetitive cystein-rich extracellular domains recognizing ligands. Functionally, TNFRSF members can be divided in three groups, according to their intracellular signal domains, containing either a death domain (DD), or a TNF receptor associated factor (TRAF) domain, or no signal domain. The first group, the “death receptors”, recruit intracellular death domain containing adaptors, such as Fas-associated DD protein (FADD) and TNFR-associated DD protein (TRADD), which activates the caspase cascade leading to apoptosis.

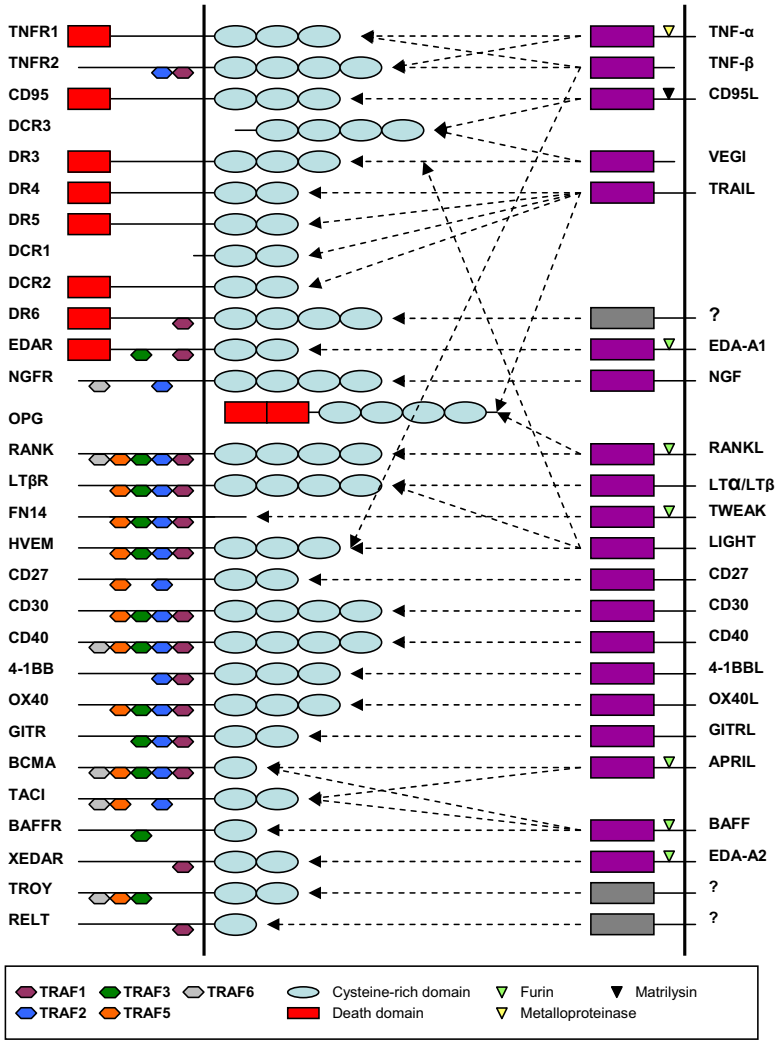

Figure 2.
A presentation of the TNF superfamily ligands and their receptors; Ligand proteins have extracellular C-terminal (type II transmembrane protein), which is known as the TNF homology domain and has 20-30% amino acid identity. Most ligands are released from cell surface by specific proteases. Receptors have N-terminal extracellular domains (type I transmembrane protein) which is characterized by the presence cysteine-rich domains. Decoy receptors (DCRs) and osteoprotegerin (OPG) work as inhibitors of signaling. TNFR; Tumor necrosis factor receptor, DR; Death receptor, EDAR; ectodysplasin-A receptor, NGFR; nerve growth-factor receptor, RANK; receptor activator of nuclear factor κB, LTβR: lymphotoxin β receptor, HVEM; herpes virus entry mediator, GITR; glucocorticoid-induced TNFR family receptor, BCMA; B-cell maturation antigen, TACI; transmembrane activator and cyclophilin ligand interactor, BAFF; B-cell activating receptor, XEDAR; X-linked EDA receptor, RELT; receptor expressed in lymphoid tissues, VEGI; vascular endothelial cell-growth inhibitor, TRAIL; TNF-related apoptosis-inducing ligand,

TWEAK; TNF-like weak inducer of apoptosis, LIGHT; lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes, APRIL; a proliferation inducing ligand, TRAF; TNFR-associated factor.

The second group mediates effects associated with cell differentiation and proliferation, by recruiting TRAF family molecules, which activate signal mediators such as mitogen activated protein kinases (MAPK), extracellular signal-related kinase (ERK), phosphatoidyl-inositol-3 kinase (PI3K) and NFκB. However, clear lines can not be drawn between the two groups; TRAF2, an activator of NFκB which induce anti-apoptotic protein synthesis, binds almost all TNFRSF members, and recent discoveries indicate that any TNF signaling simultaneously activates both apoptotic and cell-survival signals, creating a balance of opposing signals that determine cell fate.[98] Thus, TRAIL (TNF-related apoptosis inducing ligand) and CD95L, two poor inducers of NFκB, induce apoptosis more rapidly than TNF-α does, whereas RANKL (receptor activator of NFκB ligand) mainly provide survival signals through NFκB. However, NFκB does not only promote anti-apoptotic signaling, it also regulates pro-apoptotic signals through regulation of death receptors (DR1-6), and death receptor ligands such as CD95L, TNF-α and TRAIL,[99-102] illustrating the complexity of TNF-mediated cell survival signaling.

The last group of receptors conducts no intracellular signaling, but are soluble decoy receptors, i.e., providing a level of regulation by competing with signal transducing receptors for ligands. Moreover, some receptors (CD27, CD30, CD40, 4-1BB, CD95, TNF receptor I [TNFRI] and TNFRII) can be found in a soluble form, with inhibitory or stabilizing effects on their corresponding ligand, further discussed below.

The TNFSF regulates immunity at several levels, e.g., organization of lymphoid architecture[103] and controlling the activity and survival of cytotoxic effector cells. Whereas they are physiologically crucial for normal responses, any inappropriate presence is harmful, and despite tight regulation, directly pathological contribution by several TNFSF ligands is described in numerous diseases. While TNF-α is an essential element in host defense, excessive TNF-α activity plays a pathogenic role in several inflammatory disorders such as inflammatory bowel disease, psoriasis and rheumatoid artithis, and therapeutic approaches that inhibit TNF-α activity (i.e., soluble TNF receptor fusion protein or anti-TNFα-antibodies) have been successful in the treatment of diseases

such as severe rheumatoid arthritis [104] and inflammatory bowel disease.[105] TNF-α is also suggested to be an important mediator of insulin resistance in obesity, thus playing a role in the development of type 2 diabetes mellitus,[106] and is implicated in several of the pathophysiological processes that are thought to be important in the progression of chronic heart failure.[107, 108] On the other hand, TNF-α is of major importance in the host defense against certain intracellular microbes such as *Mycobacterium tuberculosis*, as illustrated in the increased frequency of mycobacterial infection in patients receiving anti-TNF therapy.[109] Another TNFSF ligand, CD40L, is associated to immunodeficiency and autoimmunity due to reduced or increased levels, respectively,[110] illustrating that both "too much" or "too little" of these ligands may be harmful.

4.3.2 TNFSF in atherosclerosis

Several TNFSF ligands have been linked to development of atherosclerosis. Experimental studies using atherosclerosis-prone apolipoprotein E deficient ($apoE^{-/-}$) mice crossed with $TNF\alpha^{-/-}$ mice, thus creating a double knockout, have shown that atherosclerotic lesion size is significantly smaller in the double knockout than in that of $apoE^{-/-}$ mice, associated with decreased expression of ICAM-1, VCAM-1 and MCP-1.[111] However, anti-atherogenic properties of TNF-α has also been reported. Thus, in TNFRI-deficient atherosclerosis-prone C75BL/6 mice fed with atherogenic diet, developed lesions were larger than wild type C75BL/6 mice, suggesting a protective role of TNFRI signaling.[112] However, *in vivo* analysis have shown TNF-α expression in the shoulder region of the plaque, associated with CD68-positive foam cells.[113] Moreover, TNF-α was expressed more abundantly in regions with a thin fibrous cap (atheromatous regions) compared to more fibrous regions, contributing to matrix degradation via MMP activation and pro-thrombogenicity by promotion of TF production in monocytes.[113, 114]

In addition to TNF-α, the TNFSF member CD40L and its receptor CD40 have attracted much attention as potential therapeutical targets of atherogenesis. The immunomodulatory dyad CD40L/CD40, present on endothelial cells, vascular SMC, macrophages, T cells and platelets within human atheroma, has been implicated in several aspects of atherogenesis and ACS.[115, 116] CD40L was originally identified on $CD4^+$ T cells, but has recently also been found on mast cells, basophils, eosinophils as well as on

activated platelets.[117] Both membrane-bound and soluble (s) forms of this ligand may interact with CD40, which is constitutively expressed on B cells, macrophages, endothelial cells and vascular SMC, resulting in various inflammatory responses with relevance to atherogenesis. Thus, *in vitro* activation of CD40L-CD40 signaling in atheroma-derived cells results in production of chemokines and other cytokines, TF, MMPs and adhesion molecules.[118, 119] *In vivo*, an important role for CD40L-CD40 interaction in the progression of atherosclerosis was demonstrated using mice deficient in CD40L and apoE showing a dramatic decrease in plaque area compared with normal apoE-deficient animals.[120] Moreover, advanced atherosclerotic lesions of these mice showed a lipid-poor collagen-rich stable plaque phenotype, with reduced macrophage and T cell content. The possible plaque stabilizing effect of CD40L neutralization was further demonstrated in another study showing that administration of anti-CD40L antibody to apoE-deficient mice induced a stable plaque phenotype both at the onset of atherosclerosis and after development of advanced plaques.[121] Recently the list of biological activities of CD40L was extended from regulation of immune responses to induction of platelet activation and thrombus formation.[122] Hence, CD40L appears to stabilize arterial thrombi by an integrin-dependent mechanism, and the absence of CD40L may delay arterial occlusion *in vivo*.[123] It is unclear whether both the transmembrane and the soluble (s) forms of CD40L are active in promoting platelet activation, but recombinant sCD40L alone was shown to enhance integrin-mediated platelet aggregation at high shear rates restoring normal thombosis in $CD40L^{-/-}$ mice.[123] Several groups have reported enhanced serum levels of sCD40L and increased expression of membrane-bound CD40L on T cells and platelets in angina patients, with particularly high levels in those with unstable disease.[115, 124] These activated platelets and T cells in unstable angina may, by providing CD40L, contribute to inflammatory reactions, MMP activation and pro-coagulant activity within an atherosclerotic plaque. Such activities may in turn further enhance platelet and T cell activation as well as thrombus formation, possibly representing a pathogenic loop promoting plaque destabilization and rupture during ACS.[115, 117] Recently, Bavendiek *et al*. suggested that CD40L derived from non-hematopoietic cell types may be of even more pathogenic importance in atherogenesis than T cell or platelet-derived CD40L.[125] Nevertheless, this ligand, which has the unique

property of both promoting inflammation and thrombus responses, activating both endothelial cells, leukocytes and platelets, operating in a self-perpetuating feedback loop, should be further investigated as a therapeutic target in atherosclerotic disorders.

5. Aims of the study

While the role of inflammation in atherosclerosis is well established, the relative importance of the various mediators, as well as their effector mechanisms, is far from clear. While several studies have focused on the role of the CD40L/CD40 dyad in atherogenesis and plaque destabilization, we hypothesized that also other members of TNFSF could be of pathogenic importance in these processes. In the present study this hypothesis was investigated by several experimental approaches including studies in well characterized patients with stable and unstable angina, studies in experimental atherosclerosis as well as in vitro studies in cells with relevance to atherogenesis such as endothelial cells, platelets, macrophages and vascular SMC.

6. Summary of results

Paper I

Enhanced T-cell expression of RANK ligand in acute coronary syndrome: possible role in plaque destabilization. RANKL, its membrane-bound receptor RANK and its soluble decoy receptor osteoprotegerin (OPG) are members of the TNFRSF. These factors have been identified as mediators of paracrine signaling in bone metabolism but are also involved in modulation of the immune response. We found that serum levels of OPG were raised in patients with unstable angina (n=40), but not in those with stable angina (n=40), compared to controls (n=20). Further, mRNA levels of RANKL were increased in T-cells in unstable angina patients accompanied by increased expression of RANK in monocytes. Strong immunostaining of OPG/RANKL/RANK was seen within thrombus material obtained at the site of plaque rupture during acute MI. Moreover, OPG/RANKL/RANK was expressed in the atherosclerotic plaques of apoE$^{-/-}$ mice, with RANKL located specifically to the plaques. *In vitro*, RANKL enhanced the release of MCP-1 in mononuclear cells from unstable angina patients, and MMP activity in vascular SMC. We propose a role of T cell mediated RANKL in the destabilization of atherosclerotic plaques, acting atherogenic by promoting inflammatory and matrix-degrading mechanisms.

Paper II

Enhanced plasma levels of LIGHT in unstable angina – possible pathogenic role in foam cell formation and thrombosis. Several inflammatory mediators including TNFSF members have been reported to increase after oxLDL stimulation of macrophages. Our results showed that the TNFSF member LIGHT (homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes) was markedly up-regulated in oxLDL-stimulated THP-1 macrophages, assessed by microarray and real time quantitative RT-PCR analysis. Underscoring these *in vitro* findings, we found enhanced plasma levels of LIGHT in angina patients, with particularly high levels in those with unstable disease. Stimulation with LIGHT enhanced lipid accumulation in

oxLDL stimulated THP-1 macrophages, probably through an up-regulation of the scavenger receptor SR-A. Moreover, upon LIGHT-stimulation, THP-1 macrophages as well as monocyte-derived macrophages were transformed into pro-thrombotic phenotypes, shown by increased expression of TF andPAI-1 as well as enhanced thrombin formation. Finally, he LIGHT-mediated enhancement of SR-A and TF expression appears to involve NF-κB activation. Our findings suggest that LIGHT could be involved in atherogenesis and plaque destabilization by promoting foam cell formation and thrombus formation.

Paper III

Inflammatory interaction between LIGHT and Proteinase-activated reseptor-2 in endothelial cells – potential role in atherogenesis. The interaction between inflammatory cytokines and endothelial cells is a critical step in atherogenesis leading to endothelial cell dysfunction and enhanced inflammation. In the present study we identified proteinase-activated receptor 2 (PAR-2) as an inflammatory mediator that was markedly enhanced by LIGHT in endothelial cells. We also found that LIGHT acted synergistically with PAR-2 activation to promote enhanced release of the pro-atherogenic chemokines IL-8 and MCP-1, underscoring the interaction between LIGHT and PAR-2 as biologically active, promoting potent inflammatory effects. We showed that the LIGHT-mediated up-regulation of PAR-2 in endothelial cells is mediated through the HVEM receptor, dependent on Jun N-terminal kinase and NF-κB intracellular signaling pathways. A LIGHT-mediated up-regulation of PAR-2 mRNA levels was also found in human macrophages. We have previously demonstrated increased plasma levels of LIGHT in unstable angina patients (paper II), and here we show a similar pattern for PAR-2 expression in peripheral blood monocytes. We also found that LIGHT, LIGHT receptors, and PAR-2 all showed enhanced expression, and in some degree co-localization in endothelial cells and macrophages, in the atherosclerotic plaques of $ApoE^{-/-}$ mice, suggesting that the inflammatory interaction between LIGHT and PAR-2 also may be operating *in vivo* within an atherosclerotic lesions. Our findings suggest that LIGHT/PAR-2 driven inflammation could represent a pathogenic loop in atherogenesis.

Paper IV

Platelets release the tumor necrosis factor superfamily ligand APRIL (TNFSF13) upon activation – increased expression of APRIL in coronary and carotid atherosclerosis. Blood platelets are regarded as important contributors to the development of atherosclerosis, not only through their role in thrombus formation, but also through their ability to promote inflammation. Thus, activated platelets release a wide range of inflammatory mediators, including members of the TNFSF (e.g., CD40L and LIGHT). In the present study we demonstrated that platelets release the TNFSF ligand *A p*rolife*r*ation-*i*nducing *l*igand (APRIL) upon thrombin receptor activation. Upon SFLLRN activation, there was a gradual release of APRIL reaching maximum after 90 minutes. This long-lasting release, which follows the same pattern as previously shown for the solubilization of CD40L and LIGHT, differ from an ordinary α-granule release where the granule components are fully released within less than 10 minutes. However, while the gradual release of APRIL is similar to the release pattern of sCD40L and LIGHT, our data show that the release of APRIL is quite differently regulated. Thus, whereas the release of LIGHT and sCD40L involves GP IIb/IIIa-dependent mechanisms and action of metal-dependent proteases as well as intracellular processes such as actin polymerization, this seems not to be the case for the release of APRIL. The release of APRIL in SFLLRN activated platelets was totally abolished when prostaglandin E_1 (PGE_1) was added to the suspension 20 minutes prior to activation. In contrast to the release of CD40L and LIGHT, PGE_1 could not modulate APRIL release when added to platelets 10 minutes after stimulation. Although APRIL mRNA in platelets was demonstrated, APRIL release was not blocked by cycloheximide, an inhibitor of protein synthesis. With relevance to atherogenesis, we found that patients with CAD had raised plasma levels of APRIL as compared with controls, and APRIL immunoreactivity was detected in aggregated platelets within the ruptured plaque in patients with STEMI and within macrophages in symptomatic carotid plaques. Our findings suggest that APRIL should be added to the list of platelet-derived soluble inflammatory proteins. The enhanced expression of APRIL in atherosclerotic disorders, both systemically and within the lesion, may suggest the involvement of APRIL in atherogenesis and plaque destabilization.

7. Discussion

7.1 Methodological considerations

7.1.1. Individuals

All patients included in this thesis had verified CAD, as assessed by coronary angiography showing at least one vessel disease (>50% narrowing of luminal diameter) and as a major objective was distinction between stable and unstable angina, a comprehensive characterization of the patient groups were conducted. Patients with unstable angina all fulfilled the criteria of Braunwald class IIIb, none had elevation of CK-MB, but some had increased levels of cardiac troponins, reflecting minimal myocardial necrosis. Average TIMI risk score for the patients with unstable angina used in paper I, II and IV was 3.13, representing a 5-13% risk of cardiovascular events (MI or death) within 14 days.[126] Enhanced inflammatory activity in these patients may reflect enhanced inflammation in the atherosclerotic plaques, or may be a consequence of minimal myocardial damage. This issue should be of consideration when evaluating measurements of circulating inflammatory markers in this patient population. The stable angina patients had stable effort angina NYHA class II or III and a positive exercise test. The diagnosis of CAD was confirmed in all patients by coronary angiography (see above), thus excluding patients with symptoms of angina pectoris without significant CAD. Standard medical therapy including β-adrenergic antagonist (83-100%), aspirin (75-92%) or warfarin, statins (80-86%) and to a lesser degree calcium antagonist (11-22%), nitrates and angiotensin-converting enzyme (ACE) inhibitors was used by the patients. According to standard procedures, unstable angina patients had received heparin or low-molecular weight heparin before inclusion, but this medication had been discontinued >12 hours before blood sampling. Nevertheless, heparin therapy may increase the plasma/serum levels of cytokines and in particular chemokines[127], but not the mRNA levels in PBMC as examined in the present study. Moreover, effect of heparin on plasma levels of cytokines seems primarily to occur within minutes or hours with no or only minor effects after 12 hours. As for most of the other medications such as statins, ACE inhibitors and inhibitors of platelet activation, down-regulatory effects on

inflammatory cytokines may be expected, resulting in underestimation rather than overestimations of differences in levels of inflammatory mediators between patients and controls. Finally, to exclude patients with causes of systemic inflammatory activation other than CAD, patients with concomitant disease such as congestinal heart failure, infections, lung diseases cancer or autoimmune disorders were not included in the studies. Also, patient with MI <6 weeks before blood sampling were excluded due to persistent systemic immune activation after such an event.

7.1.2 Isolation and culturing of cells from peripheral blood

A main focus in the present thesis was the study of mononuclear cells from peripheral blood, i.e., peripheral blood mononuclear cells (PBMC), T cells and monocytes. A problem with such studies can be *ex vivo* activation of cells during the isolation procedures. First, contamination of collection tubes, buffers and media with endotoxins and microorganisms may activate the cells and induce cytokine expression. Second, the blood sampling technique and differences in blood withdrawal speed as well as isolation of PBMC by density gradient centrifugation over Ficoll-Hypaque may influence the gene expression of cytokines in these cells. In the present study, T cells and monocytes were isolated from PBMC using well established methods that minimize *ex vivo* activation. Thus, T-cells were isolated using negative selection, while monocytes were isolated using CD14-labeled paramagnetic microbeads. This positive selection of monocytes could potentially induce cell activation via CD14 engagement, but the use of microbeads and low temperature (i.e., 4ºC) during the separation procedure have been shown to promote negligible monocyte activation. To further assure minimal differences in *ex vivo* activation in the isolation of cells from patients and healthy controls, all blood sampling and cell isolation protocols were standardized and cell isolation was performed under sterile conditions and only sterile and endotoxin-tested (endotoxin-levels< 10 pg/ml) materials were used. After isolation, the cells were snap-frozen and stored on liquid nitrogen, or used directly in cell culture experiments. Notably, gene expression of cytokines known to be promptly up-regulated after various forms of activation such as hypoxia (e.g., IL-8 and TNF-α) were only weakly expressed in isolated cells from healthy

control subjects, indicating that any differences in gene expression observed in patients are related to *in vivo* and not *ex vivo* activation.

7.1.3 Measuring of cytokines in blood

Factors related to blood collection and processing that may influence the measured cytokine levels in plasma and serum include procedure for blood collection, contamination of collection tubes, buffer and media, time before storage, temperature during storage and freeze thaw cycles.[128, 129] However, the effect of these influencing factors may be reduced by using sterile and endotoxin-free collection tubes, buffers and media, immediate execution of separation protocols and freezing, and by freezing and thawing the samples less than three times.[128, 129] In the present study we consistently adhered to these standards. Moreover, the measured levels of cytokines may be affected by the choice of anti-coagulant (plasma), and the release of cytokines from platelets during *ex vivo* coagulation (serum). In general, it has been reported that recovery of cytokines is more optimal from plasma than from serum, and that EDTA should be preferred as an anti-coagulant as it seem to inhibit *ex vivo* cytokine production during plasma preparation.[128, 129] Furthermore, examination of cytokines that may be present in platelets, such as LIGHT and APRIL should be performed in platelet-poor plasma, to avoid release of cytokines from platelets *ex vivo* during the thawing procedure. In this work cytokine measurments were performed according to these precautions.

7.1.4 Enzyme Linked Immunosorbent Assays (ELISAs)

Commercially available ELISAs were used for measuring the protein levels of cytokines in this study. ELISAs are widely used as they are easy, time sharing and specific, but some considerations should be mentioned. First, immunoassays may detect both active and inactive forms of a protein, potentially giving the wrong impression of ongoing biological processes. Second, the recognition profile of the antibodies will vary between ELISA kits and manufacturers. Third, the presence of soluble cytokine receptors, cytokine antibodies and binding proteins in the biological sample might affect the measured cytokine levels. In this study, data were only compared when using the same ELISA kits from one manufacturer. Further, when comparing groups (e.g., controls vs.

stable and unstable angina patients), all groups were represented on any microtiter plate to minimize run-to-run variability when interpreting group-to-group variations.

7.1.5 mRNA measurements

RNA was isolated using silica-gel based membranes (RNeasy columns) or by MagNA Pure LC robot. RNA concentration and purity was evaluated by measuring the absorbance at 260/280 and 260/230 nm using nanodrop spectrophotometer. Only samples of high integrity and purity were further analyzed. For quantification and comparison of the mRNA transcripts in question, we used real time quantitative reverse transcriptase PCR (qPCR). This method is based on a fluorogenic dye (or sequence specific probe) which emits detectable fluorescence proportionally to the increasing amount of PCR-products produced in a 40 cycle reaction. Gene expression was for each sample normalized to β-actin, which at least theoretically is stably expressed in a wide range of conditions and cell types, thus obtaining an intra-sample relative expression value for the gene in question. Sample-to-sample variations were minimized by loading approximately equal amounts of RNA into the reverse transcriptase reaction. Further, to avoid misinterpretation of data because of assay-to-assay variations, all samples from one experiment were analyzed in one assay. By using cDNA microarray we were able to analyze expression of several thousand genes simultaneously. This technique has limitations in reliability related to data collection, analysis and validation, therefore used for hypothesis generation. Candidate genes were identified by this method in paper III and IV in this thesis, and all gene expression data derived from microarrays were confirmed by qPCR.

7.2 RANK ligand in atherosclerosis

7.2.1 RANK ligand and T cells

In paper I, we showed enhanced expression of RANKL in T cells from patients with unstable angina. A pathogenic role of T cells in atherosclerosis is well established, and some recent studies suggest that this also may involve certain members of the TNF superfamily as co-stimulators for T cell activation.[130] Our findings in the present study suggest that RANKL-RANK interaction involving T cell-related mechanisms also could

be involved in atherogenesis. RANKL expression has previously been detected in activated T cells, and as a key regulator of bone metabolism. In fact, RANKL is highly expressed by osteoblast/stromal cells, and essential in osteoclast activation. However, T cell-derived RANKL has also been demonstrated to modulate immune responses, lymphocyte maturation and lymph node organogenesis.[131-137] RANKL signals through its transmembrane receptor RANK, which is mainly expressed by monocyte/macrophage lineage cells, including dendritic cells, osteoclasts and their precursors, to generate multiple intracellular signals that affect cell differentiation, function and survival.[138-141] Kanamaru *et al* showed that RANKL was rapidly shed by the metalloprotease-disintegrin TNF-α convertase (TACE), or a related metalloprotease, from the T cell membrane in both *in vitro* activated T cells and from T cells in inflammatory tissues (rheumatoid synovium).[137, 142] In paper I, we documented strong immunostaining of RANKL in atherosclerotic plaque, and in thrombus material. Although its receptors RANK and OPG were found in healthy vessel wall, RANKL was specifically related to the plaque, and associated to the T cell and macrophage-rich core region. Moreover, we showed that mRNA expression of the signal-transducing RANKL receptor, RANK, was significantly higher in monocytes from patients with unstable angina, and non-significantly increased in T cells from the same patient group. Taken together, these findings are indicative for paracrine signaling by T cells of the atherosclerotic plaque, involving RANKL-RANK interaction, with consequences discussed below.

7.2.2 RANKL and its soluble receptor osteoprotegerin (OPG)

Soluble TNF receptors are typically considered to act as inhibitory regulators of TNF-signaling, by competing with signal-transducing cell surface TNF receptors. OPG is a soluble decoy receptor binding RANKL and TRAIL. In bone, RANKL promotes the resorptive activity and survival of mature osteoclasts, and OPG counteracts these RANKL-RANK mediated actions by binding RANKL, and the RANKL-to-OPG balance critically determinate bone remodeling and net bone mass. However, as discussed above, the effect of RANKL-RANK interaction is not restricted to bone metabolism. Hence, the RANKL/RANK/OPG axis has been discussed as the molecular link between skeletal, immune and vascular diseases,[143] and raised circulating levels of OPG are reported in

CAD patients with increasing levels according to disease severity.[144] OPG levels have also been shown to be a reliable predictor of forthcoming cardiovascular events in various study populations.[144-147] In paper I, we showed significantly raised serum levels of OPG in unstable but not in stable angina, further supporting these findings. Strong immunostaining of OPG in vulnerable atherosclerotic plaques further implicate the RANKL/RANK/OPG axis in atherogenesis. These findings may also suggest that the reliability of OPG as a marker of atherosclerotic disorders could reflect that plasma/serum levels, at least partly, are derived from the atherosclerotic lesion. Furthermore, while primary thought to inhibit the binding of RANKL to its receptor RANK, we show in paper I that OPG may also enhance the RANKL-inducing effects on MMP activity in vascular SMC, at least under high RANKL/OPG ratio. A similar stabilizing or enhancing effects on its corresponding ligand has also been demonstrated for soluble TNF-Rs.[148-151] Moreover, we also found that OPG had MMP-inducing effects in SMC on its own, further challenging the view that OPG is solely a RANKL inhibitor. The ability of OPG to induce MMP activity and in particular MMP-9, has recently been supported by others.[152]

7.2.3 RANKL and plaque destabilization

Several findings point at the RANKL/RANK/OPG axis as a contributor to plaque destabilization. First, increased RANKL expression on T cells was prominent in the unstable patient group. Second, Golledge *et al* reported OPG to be expressed at higher levels in symptomatic than in asymptomatic atherosclerosis,[153] and we extended these findings by demonstrating enhanced RANKL immunostaining in plaques from apoE$^{-/-}$ xCD4dnTßRII mice (i.e., ApoE$^{-/-}$ mice that are lacking the TGF-ß receptor on T cells), which develop a more severe atherosclerotic phenotype relative to apoE$^{-/-}$ mice. These findings were supported by strong immunostaining of the RANKL/RANK/OPG triad in thrombus material obtained from the site of plaque rupture in patients undergoing PCI. Third, during PCI, a mechanically induced plaque rupture, we found increased RANKL expression in PBMC, further suggesting that increased RANKL expression is a feature of unstable disease. Moreover, the RANKL-induced MCP-1 release was significantly elevated in PBMC from unstable angina as compared to stable angina patients and

healthy controls, potentially reflecting enhanced RANK expression on monocytes in unstable patients. Chemotactic properties of RANKL on monocytes have previously been reported by others, acting additively to MCP-1.[154] We further showed that RANKL and OPG induced MMP activity in SMC, both alone and in a cooperative manner. Taken together, a functional, pathogenic picture emerges, where the RANKL/RANK/OPG axis could contribute to some of the hallmark features of plaque destabilization, namely recruitment of inflammatory cells and degradation of the fibrous cap. However, RANKL has also been shown to protect endothelial cells against apoptosis,[155] a property with potential anti-atherogenic properties. Moreover, studies in *OPG*$^{-/-}$apoE$^{-/-}$ mice as well as in LDL receptor (LDLR)$^{-/-}$ mice treated with recombinant OPG suggest a role for OPG in vascular calcification rather than plaque stability,[152, 156] underscoring that these issues will have to be further clarified, including studies in apoE$^{-/-}$ or LDLR$^{-/-}$ mice over-expressing or lacking RANKL or RANK.

7.3 A proliferation inducing ligand (APRIL) in atherosclerosis

7.3.1 Platelet-mediated inflammation

Several lines of evidence support a role for platelets as inflammatory cells. Platelets provide a wide range of growth factors and inflammatory mediators which are released mainly from the intracellular storage granules upon activation. These include several chemokines such as chemokine platelet factor-4 (PF-4), β-thromboglobulin, Regulated upon Activation Normal T cell Expressed and Secreted (RANTES), growth-regulated oncogene-α (GRO- α), epithelial neutrophile activating peptide-78 (ENA-78) and neutrophil activating peptide (NAP-2) as well as IL-7, a cytokine with the ability to induce T cell activation without the presence of antigens.[96, 157-160] These soluble ligands may together with membrane-bound mediators expressed on the platelet surface upon activation (i.e., P-selectin), promote inflammatory responses in adjacent monocytes involving NFκB activation. Moreover, recent studies suggest that platelets also may modulate the function of other leukocyte subsets (i.e., natural killer cells, granulocytes, T cells), and they have been found to enhance chemotactic and adhesive properties of endothelial cells as well as IL-1 production in vascular SMC.[161, 162] Finally, platelets may themselves respond to inflammatory mediators. In fact, platelets have recently been

found to express several chemokine receptors that upon stimulation enhance platelet activation.[163] Hence, during activation platelets may not only promote thrombus formation, but seem also to release and express inflammatory mediators, induce an inflammatory response within leukocytes and endothelial cells, and respond with activation to several of the inflammatory mediators produced by these cells. Such platelet-mediated inflammation seems to play a pathogenic role in atherosclerosis as well as in other inflammatory disorders. Thus, platelets co-localize with monocytes in early stages of atherosclerosis,[164] and platelet-leukocyte interaction appears also to be involved in plaque destabilization during ACS as well as in the pathogenesis of various bacterial infections and various forms of vasculitis.[165-167]

7.3.2 TNF superfamily and platelet-mediated inflammation

Several members of the TNF superfamily are also reported to be expressed by platelets, and to date, CD40L, FasL, TRAIL and LIGHT are described.[122, 168-170] While the biological activity of platelet-derived soluble TNFSF members has been an issue of controversy, membrane-bound CD40L on platelets is shown to be involved in initiation of an inflammatory response at the vessel wall by inducing adhesion molecules and secretion of chemokines in the vascular endothelial cells.[122, 165, 171] Some studies have also suggested biological roles for sCD40L. Thus, Zhang *et al* reported that sCD40L released from platelets is able to activate fibroblasts *in vitro*, as indicated by regulation of cyclooxygenase (COX)-2 expression.[172] Aukrust *et al* reported that serum-derived sCD40L could induce MCP-1 in PBMC, and these authors have also showed the ability of sCD40L to promote inflammatory and pro-thrombotic responses in endothelial cells.[115, 166, 173] Moreover, André and colleagues demonstrated that recombinant sCD40L alone could enhance integrin-mediated platelet aggregation at high shear rates restoring normal thrombosis in $CD40L^{-/-}$ mice.[123] These authors have more recently suggested that sCD40L is a ligand for GP IIb-IIIa inducing platelet stimulation as evidenced by the generation of platelet microparticles and by the activation of the receptor function of GP IIb-IIIa for soluble fibrinogen, whereas secretion from α- and dense granules was not observed.[174] As for platelet derived FasL, both membrane bound and soluble forms have been reported, but so far, its biological effect has been restricted to the membrane-bound

form promoting apoptosis in Fas-positive cancer cells.[168] While surface expression and release of TRAIL from activated platelets are documented, [169] no biological functions of platelet-derived TRAIL have been described yet. Recently, Otterdal et al showed that LIGHT was released from platelets upon activation.[170] This study also showed that platelet-derived soluble LIGHT was able to induce IL-8 and MCP-1 release from endothelial cells as well as monocytes. In paper IV we demonstrate that APRIL, another member of the TNF superfamily, is released by platelets upon activation.

7.3.3 Platelet-derived APRIL

In contrast to the rapid release of α-granule contents from activated platelets, APRIL was released in a gradual and long lasting manner, showing a similar release pattern as previously described for other TNFSF members (e.g., CD40L and LIGHT).[170, 175-177] López-Fraga *et al* demonstrated that APRIL is synthesized as a transmembrane protein and cleaved to its soluble and biologically active form by furin, a pro-protein convertase. Further, using human embryonic kidney (HEK) 293T cells, these authors demonstrated that processing of APRIL occurs in the golgi apparatus and is subsequently released as a soluble protein.[178] In platelets however, blocking experiments using both membrane permeable and impermeable furin inhibitors did not affect APRIL release upon activation. Further, brefeldin A, a golgi-disturbing agent, did not block the release of APRIL upon platelet activation. Platelets contain megakaryocytic mRNA and ribosome apparatus, and platelet protein synthesis is previously described.[179] We detected APRIL mRNA transcripts in purified platelets, but inhibition of translation using cycloheximide did not attenuate APRIL release from activated platelets. We have previously shown that inhibitors of calcium dependent MMPs and disintegrin and metalloproteinases (ADAMs) (i.e., EDTA [a strong calcium chelator] and GM6001 [a hydroxamate inhibitor of MMPs], and inhibitors of actin polymerization (cytochalasin D) markedly attenuate the platelet-mediated release of sCD40L and LIGHT.[170, 177] However, none of these substances influenced the release of APRIL in SFLLRN activated platelets, even when added to PRP 20 minutes before activation. Moreover, in contrast to our previous reports on sCD40L and LIGHT, Glanzmann's thrombasthenia, an inherited deficiency of GP IIb/IIIa, did not influence the release of APRIL in SFLLRN activated PRP. Prostaglandin

E_1 (PGE_1) is an effective platelet inhibitor, acting through its ability to raise intracellular cAMP levels and thereby decreasing intracellular Ca^{2+} levels. We were able to block APRIL release by incubating the platelets with PGE_1 for 20 minutes prior to SFLLRN activation. This blocking effect was however not achieved when adding PGE_1 15 minutes after platelet activation, as we have previously shown for sCD40L and LIGHT,[170, 177] indicating an exclusive release mechanism for APRIL compared to other TNFSF ligands.

7.3.4 Physiologic and pathophysiologic effects of APRIL

APRIL and its closely related B cell-activating factor of the TNF family (BAFF), have several features in common, they are both expressed in monocytes and macrophages, and both participate in stimulation of peripheral B cell and plasma cell survival and proliferation by binding to their respective receptors; APRIL binds B cell maturation antigen (BCMA) and proteoglycans, BAFF binds BAFF-receptor (BAFFR), whereas both BAFF and APRIL binds transmembrane activator and CAML interactor (TACI).[180-182] The relative importance of BAFF and APRIL in immune responses are largely unknown,[181] but interestingly, we showed increased levels of APRIL but not BAFF (Sandberg WJ, Aukrust P, unpublished data) in plasma from angina patients.

APRIL is a known regulator of normal B cell development and function.[183] It has also been shown to promote T cell activation as well as to induce the up-regulation of several key co-stimulatory molecules on B cells, and thereby significantly enhancing their antigen presentation function.[183] Recent studies have suggested an important role for APRIL, not only in normal immune responses, but also in the establishment and/or maintenance of autoimmune and inflammatory diseases. Thus, altered circulatory levels of APRIL have been reported in several inflammatory disorders. Koyama *et al* reported raised serum APRIL in systemic lupus erythematosus,[184] and both APRIL and BAFF levels are associated to Sjogren's syndrome.[185, 186] APRIL levels were also elevated in synovial fluid from patients with inflammatory arthritis.[187] However, APRIL levels within the inflamed synovia did not correlate to serum levels, indicating that APRIL may be produced and potentially play an active role at the sites of inflammation.

In paper IV we show that patients with CAD had raised plasma levels of APRIL as compared with healthy controls, and with relevance to the role of platelet-derived

APRIL, APRIL immunoreactivity was found in aggregated platelets within the ruptured plaque in STEMI patients. Moreover, strong APRIL immunostaining was found within macrophages in symptomatic carotid plaques. The pathogenic consequences of this finding is at present unclear, but based on its ability to enhance T cell activation as well as to down-regulate apoptosis in these cells, promoting an long-lived inflammatory phenotype,[183] it is tempting to hypothesize that APRIL could contribute to T cell-related inflammation within the atherosclerotic lesion.

Several studies support a role of APRIL in B cell function, converting subliminal B cell activation to a productive response.[183] However, while the role of macrophages and T cells in atherogenesis is well established, the role of B cells is less studied. Only a few B cells are detected in atherosclerotic plaques, but substantial amounts of B cells and plasma cells are found in periadventitial lymphoid infiltrates surrounding advanced lesions.[188, 189] Several lines of evidence support the hypothesis that humoral immunity protects against atherosclerosis. Thus, splenectomy (i.e*.,* removal of the B cell–rich spleen) increases lesion development in apoE$^{-/-}$ mice, whereas B cell transfer from atherosclerotic to splenectomized apoE$^{-/-}$ mice ameliorates atherosclerosis.[190] These effects may depend at least partly upon the production by B cells of antibodies against candidate antigens, including oxLDL.[191] On the other hand, it has also been suggested that B cells could promote inflammatory responses within an atherosclerotic lesion through production of antibodies and inflammatory cytokines. Future studies addressing the role of B cells in atherogenesis should also investigate the ability of APRIL to modulate these responses, being expressed at high levels within the atherosclerotic lesion. Future studies should also investigate the ability of APRIL to modulate the phenotype of other cells with relevance to atherosclerosis such as macrophages and dendritic cells. Its ability to bind and potentially act through heparan sulfate proteoglycans[192] is intriguing in relevance to its potential role in atherogenesis.

From a therapeutical point of view, it should be underscored that although most of the CAD patients used aspirin as platelet inhibition, they still had raised plasma levels of APRIL (paper IV). Moreover, GP IIb/IIIa blocking seems not to influence the release of APRIL in activated platelets, and clopidogrel administration does not modify plasma levels of APRIL in CAD patients (Sandberg WJ, Otterdal K, and Aukrust P, unpublished

data). Based on our *in vitro* findings, PGE_1 and their analogs could potentially inhibit platelet-mediated release of APRIL in CAD patients, but the clinical use of these compounds for the treatment of cardiovascular diseases is restricted due to unwanted side effects.[193] If a pathogenic role of APRIL in atherogenesis and plaque destabilization is confirmed in forthcoming studies, blocking of APRIL effects or release could be investigated as a therapeutic approach in these patients. Blockade of APRIL with TACI-Ig in a mouse model of systemic lupus erythematosus leads to a significant inhibition of disease signs and symptom.[194] Based on the large number of literature citations linking APRIL/BAFF and their receptors to various diseases, and the resulting interest of at least seven biotechnology companies, there is abundant optimism that neutralizing APRIL and/or BAFF will prove to be an effective therapy for patients suffering from a variety of autoimmune diseases and cancers,[183] and potentially also atherosclertic disorders.

7.4 The role of LIGHT in atherosclerotic disorders

7.4.1 LIGHT - HVEM signaling in atherogenesis

The TNFSF member LIGHT have a wide tissue distribution and cell-context specific functions due to interaction with two different receptors, the herpes virus entry mediator (HVEM) and/or the lymphotoxin β receptor (LTβR).[195, 196] LIGHT can also bind to a soluble receptor, soluble decoy receptor 3 (DcR3), and although it has been shown to suppress LIGHT-mediated apoptosis *in vitro*,[197] its biological function is unclear. Both LTβR and HVEM also have a wide tissue distribution, and in most cases, HVEM-mediated signaling confer anti-apoptotic or growth-inducing properties, and define LIGHT as a co-stimulatory activator of T cells and dendritic cells, whereas LIGHT-LTβR signaling typically induce apoptosis or cytokine production, or organization of lymphoid structures.[103, 198-202] LIGHT-HVEM signaling deserve particular focus in atherogenesis. First, Lee *et al* showed strong immunostaining of LIGHT and foam cell-associated HVEM in atherosclerotic carotid plaques,[113] and in paper III we found some degree of co-localization of LIGHT and HVEM within the atherosclerotic lesion of $apoE^{-/-}$ mice, primarily located to the areas of endothelial cells and macrophages. Second, HVEM is highly expressed in peripheral lymphocytes, dendritic cells and

macrophages,[203] and the LIGHT-mediated induction of proatherogenic mediators such as TNF-α, IL-8 and MMP in monocytes and macrophages could potentially involve HVEM signaling pathways. Third, the ability of LIGHT to promote T cell activation in a HVEM dependent and CD28 independent manner, preferentially inducing inflammatory Th1 responses, further support a role for LIGHT-HVEM interaction in atherogenesis.[204, 205] Further, indicative data of vascular LIGHT-HVEM signaling was obtained in mice undergoing heart transplantation; injection of HVEM-Ig attenuated progression of vessel luminal occlusion, suggesting that the LIGHT-HVEM pathway is involved in the development of graft-related vasculopathy.[206] Wei *et al* demonstrated that LIGHT-HVEM signaling stimulated macrophage migration and promoted vascular SMC proliferation, strengthening the hypothesis of a pathogenic LIGHT-HVEM-mediated interaction in atherosclerotic disorders.[207] Recently, Cai *et al* showed that CD160, a glycosylphosphatidylinositol-anchored member of the immunoglobulin superfamily, serves as a negative regulator of $CD4^+$ T cell activation through its interaction with HVEM.[208] However, at present there are no data on the relative importance of CD160 and LIGHT in the HVEM-mediated T cell responses in various clinical disorders including atherosclerosis.

7.4.2 LIGHT and proteinase-activated receptor-2 (PAR-2)

The interaction between inflammatory mediators and vascular endothelium is an important step in atherogenesis and LIGHT has been demonstrated to induce expression of inflammatory molecules in endothelial cells. Chang *et al* showed that LIGHT could upregulate ICAM-1 and VCAM-1 expression, leading to increased monocyte adhesion.[209] Also, LIGHT was shown to induce expression of PGI_2, GRO-α and COX-2, mediated through the HVEM receptor, whereas IL-8 was shown to be induced by LIGHT through LTβR using a LTβR-specific LIGHT mutein. [209] We demonstrated additional pro-inflammatory effects of LIGHT in endothelial cells and macrophages by showing a LIGHT-mediated increase of proteinase-activated receptor-2 (PAR-2) expression (paper III). In line with its role in inflammatory responses, recent studies suggest a pathogenic role of PAR-2 activation in various inflammatory disorders such as arthritis [210, 211] and a number of skin disorders.[212, 213] In contrast, other studies have provided evidence for a

protective, anti-inflammatory role in airways [214-216] and intestine.[217, 218] These studies indicate that PAR-2 exhibits a duality of function depending at least partly upon the tissue and the disease context. In paper III, we demonstrated that LIGHT-HVEM interaction in vascular endothelium promoted PAR-2 expression through activation of the c-JUN N-terminal kinase (JNK) intracellular signaling pathway. LIGHT stimulation of LTβR is a potent inducer of NFκB activation with subsequent induction of a wide range of inflammatory responses.[219] Although we in paper III confirmed NFκB activation in LIGHT stimulated HUVEC, our blocking experiments strongly suggest that the LIGHT-mediated induction of PAR-2 in endothelial cells involves the JNK signaling pathway, and seems not to be dependent on NFκB activation. Although LIGHT-LTβR signaling recently has been shown to involve JNK activation in fibroblasts.[220] overexpression of HVEM seems to be of particular importance for the LIGHT-mediated activation of JNK.[221]

In paper III, we also showed that LIGHT acted synergistically with PAR-2 activation to promote an inflammatory response in endothelial cell, suggesting that LIGHT-mediated up-regulation of PAR-2 is biological relevant. Thus, whereas the PAR-2 agonist peptide SLIGKV alone only moderately promoted release of IL-8 and MCP-1 from endothelial cells *in vitro*, co-stimulation with LIGHT increased cytokine-release more than two-fold compared to LIGHT-stimulation alone, and five- to ten-fold compared to PAR-2-stimulation alone. This finding gives support to the notion that endothelial-related PAR-2 primarily plays an inflammatory role. In paper II, we showed that angina patients had increased plasma levels of LIGHT with particularly high levels in those with unstable disease. In paper III we report a same pattern for PAR-2 expression in monocytes. We demonstrated the ability of LIGHT to induce PAR-2 expression in monocyte-derived macrophages *in vitro*. Although we have no *in vivo* data on this issue in angina patients, it is tempting to hypothesize that these findings may reflect that an inflammatory interaction between LIGHT-PAR-2 also may be operating in vivo in angina patients. The demonstration of enhanced expression of PAR-2, LIGHT, and its receptors within atherosclerotic lesions of apoE$^{-/-}$ mice, at least partly co-localized in area of macrophages and in particular of endothelial cell, further support the *in vivo* relevance of our findings. Although further studies clearly are needed, our findings suggest that

LIGHT/PAR-2 driven inflammation, involving HVEM and JNK-mediated signaling, could represent a novel pathogenic loop in atherogenesis and plaque progression, potentially representing a target for therapy in this disorder.

7.4.3 LIGHT and lipid metabolism

LDL and its modified versions, such as oxLDL, play fundamental roles in the development of atherosclerosis, as described above. In paper II, we identified LIGHT as a possible important mediator of inflammatory response to oxLDL in foam cell macrophages, by showing enhanced LIGHT expression in THP-1 macrophages in response to oxLDL-stimulation. Moreover, we demonstrated that LIGHT promoted foam cell formation at least partly by its ability to up-regulate scavenger receptor-A (SR-A) expression in macrophages. Moreover, the LIGHT-mediated enhancement of SR-A expression was accompanied by enhanced of acetylated LDL, a modified form of LDL that preferentially bind SR-A,[222] further supporting the LIGHT-mediated up-regulation of SR-A in foam cell formation. Experimental studies have previously established that lipid uptake is correlated to the level of SR-A expression in macrophages, [223, 224] and SR-A inhibition is shown to reduce atherosclerosis. [223, 225, 226] It is well known that lipids and in particular oxLDL may promote inflammatory responses within an atherosclerotic lesion. However, inflammatory mediators could also modulate lipid metabolism and our finding in paper II suggest that this immune-meditated lipid accumulation could involve LIGHT-mediated mechanisms. A recent study by Lo *et al*. further supports such a notion.[227] These authors identified LIGHT as a critical regulator of lipid metabolism by showing that over-expression of LIGHT in T cells regulated (i.e., down-regulation of hepatic lipase) hepatic gene transcription and induced hypertriglyceridemia and hypercholesterolemia through LIGHT-LTβR signaling in transgenic mice. They also demonstrated that blocking LIGHT-LTβR signaling significantly reduced cholesterol levels in hypercholesterolemic LDLR-deficient mice. Our findings in paper III, showing that LIGHT signaling might interfere with peroxisome-proliferator activator (PPAR)-γ activation further support a link between lipid metabolism and LIGHT.

7.4.4 LIGHT and thrombus formation

While platelet activation and pro-thrombotic stimuli (e.g., PAR-1 activation) may cause inflammation, inflammation may also cause thrombus formation. In paper II, we reported for the first time potentially pro-thrombotic properties of LIGHT in human macrophages. Levels of TF content and macrophages are strongly correlated in the lipid rich areas of atherosclerotic lesions, representing an important pro-thrombotic feature of the lesion. Previously, TNFSF members such as CD40L and TNF-α have been shown to enhance TF expression in these cells,[116, 228, 229] and in paper II we show that LIGHT could exhibit similar properties. Thus, we demonstrated increased TF content in cell lysate from LIGHT stimulated THP-1 macrophages, accompanied by increased plasminogen activator inhibitor (PAI)-1 expression, indicating that LIGHT promote thrombin formation and impair fibrinolytic activities. This was further underscored by a LIGHT-mediated enhancement thrombin formation as assessed by F1+2 levels in THP-1 supernatants after adding human plasma to cell cultures. Moreover, the thrombin receptor agonist peptide SFLLRN induced LIGHT expression in THP-1 macrophages suggesting a possible pathogenic loop promoting thrombus formation, involving interactions with LIGHT, thrombin and macrophages.

Our finding show that LIGHT may transform macrophages into a prothrombotic phenotype, and our group has recently shown that a similar mechanism could be operating in endothelial cells. Thus, LIGHT stimulation of HUVEC did not only induce the release of the prothrombotic and antifibrinolytic mediators TF and PAI-1, respectively, but also decreased endothelial production of its natural anticoagulants (i.e., thromomodulin) further underscoring the pro-thrombotic potential of LIGHT. Taken together, our findings in paper II and III, suggest that LIGHT could serve as a molecular link between lipid metabolism, inflammation and thrombus formation, feature that all take place in atherosclerotic plaque.

8. Conclusions

The main conclusions presented in this thesis can be extracted as follows:

- We show enhanced expression of the OPG/RANKL/RANK system both in clinical and experimental atherosclerosis, with enhanced T cell expression of RANKL as an important feature of unstable disease. Enhanced RANKL expression could contribute to atherogenesis and plaque destabilization at least partly by promoting chemokine release and matrix degradation. Our observations also suggest that OPG under certain circumstances could enhance rather than attenuate the MMP-inducing effects of RANKL.
- Angina patients had markedly increased plasma levels of LIGHT with particularly high levels in those with unstable disease. This raised LIGHT level could at least partly involve interactions between oxLDL and macrophages. LIGHT was found to promote lipid accumulation as well as the expression of TF and PAI-1 in macrophages, transforming these cells into a pro-thrombotic and foam cell phenotype.
- The inflammatory interaction between LIGHT and PAR-2 in endothelial cells, involving HVEM and JNK signaling pathways, could represent a novel pathogenic loop in atherogenesis and plaque progression contributing to vascular inflammation within the atherosclerotic lesion.
- Platelets contain the TNFSF member APRIL, which is released upon platelet activation in a gradual manner different from the rapid release of α-granule content. Although the kinetic may seem similar to that of other platelet-derived TNF members, the regulation of APRIL release is different but that of sCD40L and LIGHT. APRIL and its corresponding receptors were expressed in thrombus material obtained at site of plaque rupture in STEMI patients as well as within symptomatic carotid plaques. Our findings suggest that APRIL should be added to the list of mediators that are involved in platelet-mediated inflammation during atherogenesis.

Our findings suggest that the OPG/RANKL/RANK axis as well as LIGHT and APRIL could be involved in atherogenesis and plaque destabilization contributing to inflammation, thrombus formation, matrix degradation and lipid accumulation. These mediators, representing molecular links between different and interacting processes during atherogenesis, could potentially represent new targets for therapy in atherosclerotic disorders.

9. References

1. Murray CJ, Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet.* 1997;349:1436-1442.

2. Chockalingam A, Balaguer-Vintro I, Achutti A, de Luna AB, Chalmers J, Farinaro E, Lauzon R, Martin I, Papp JG, Postiglione A, Reddy KS, Tse TF. The World Heart Federation's white book: impending global pandemic of cardiovascular diseases: challenges and opportunities for the prevention and control of cardiovascular diseases in developing countries and economies in transition. *Can J Cardiol.* 2000;16:227-229.

3. Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong Y. Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.* 2007;115:e69-171.

4. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med.* 2005;352:1685-1695.

5. Yusuf S, Reddy S, Ounpuu S, Anand S. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. *Circulation.* 2001;104:2746-2753.

6. Steinberg D. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat Med.* 2002;8:1211-1217.

7. Davies MJ. The composition of coronary-artery plaques. *N Engl J Med.* 1997;336:1312-1314.

8. Lusis AJ. Atherosclerosis. *Nature.* 2000;407:233-241.

9. Goldman L, Hashimoto B, Cook EF, Loscalzo A. Comparative reproducibility and validity of systems for assessing cardiovascular functional class: advantages of a new specific activity scale. *Circulation.* 1981;64:1227-1234.

10. Braunwald E. Unstable angina. A classification. *Circulation.* 1989;80:410-414.

11. Hamm CW, Braunwald E. A classification of unstable angina revisited. *Circulation.* 2000;102:118-122.

12. Montalescot G, Dallongeville J, Van Belle E, Rouanet S, Baulac C, Degrandsart A, Vicaut E. STEMI and NSTEMI: are they so different? 1 year outcomes in acute myocardial infarction as defined by the ESC/ACC definition (the OPERA registry). *Eur Heart J.* 2007;28:1409-1417.

13. Risk of myocardial infarction and death during treatment with low dose aspirin and intravenous heparin in men with unstable coronary artery disease. The RISC Group. *Lancet.* 1990;336:827-830.

14. Cairns JA, Gent M, Singer J, Finnie KJ, Froggatt GM, Holder DA, Jablonsky G, Kostuk WJ, Melendez LJ, Myers MG, et al. Aspirin, sulfinpyrazone, or both in unstable angina. Results of a Canadian multicenter trial. *N Engl J Med.* 1985;313:1369-1375.

15. Juul-Moller S, Edvardsson N, Jahnmatz B, Rosen A, Sorensen S, Omblus R. Double-blind trial of aspirin in primary prevention of myocardial infarction in

patients with stable chronic angina pectoris. The Swedish Angina Pectoris Aspirin Trial (SAPAT) Group. *Lancet.* 1992;340:1421-1425.

16. Theroux P, Ouimet H, McCans J, Latour JG, Joly P, Levy G, Pelletier E, Juneau M, Stasiak J, deGuise P, et al. Aspirin, heparin, or both to treat acute unstable angina. *N Engl J Med.* 1988;319:1105-1111.

17. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet.* 1996;348:1329-1339.

18. More RS, Chauhan A. Antiplatelet rather than anticoagulant therapy with coronary stenting. *Lancet.* 1997;349:146-147.

19. Topol EJ. A guide to therapeutic decision-making in patients with non-ST-segment elevation acute coronary syndromes. *J Am Coll Cardiol.* 2003;41:S123-129.

20. Yeghiazarians Y, Braunstein JB, Askari A, Stone PH. Unstable angina pectoris. *N Engl J Med.* 2000;342:101-114.

21. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet.* 1994;344:1383-1389.

22. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med.* 1998;339:1349-1357.

23. Pepine CJ. Optimizing lipid management in patients with acute coronary syndromes. *Am J Cardiol.* 2003;91:30B-35B.

24. Pitt B, Mancini GB, Ellis SG, Rosman HS, Park JS, McGovern ME. Pravastatin limitation of atherosclerosis in the coronary arteries (PLAC I): reduction in atherosclerosis progression and clinical events. PLAC I investigation. *J Am Coll Cardiol.* 1995;26:1133-1139.

25. Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, Braunwald E. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med.* 1996;335:1001-1009.

26. Schwartz GG, Olsson AG, Ezekowitz MD, Ganz P, Oliver MF, Waters D, Zeiher A, Chaitman BR, Leslie S, Stern T. Effects of atorvastatin on early recurrent ischemic events in acute coronary syndromes: the MIRACL study: a randomized controlled trial. *Jama.* 2001;285:1711-1718.

27. Boren J, Gustafsson M, Skalen K, Flood C, Innerarity TL. Role of extracellular retention of low density lipoproteins in atherosclerosis. *Curr Opin Lipidol.* 2000;11:451-456.

28. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* 1993;362:801-809.

29. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med.* 1989;320:915-924.

30. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation.* 2001;104:365-372.
31. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis.* 1986;6:131-138.
32. Batra V, Musani AI, Hastie AT, Khurana S, Carpenter KA, Zangrilli JG, Peters SP. Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)-beta1, TGF-beta2, interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on alpha-smooth muscle actin and collagen III synthesis by primary human lung fibroblasts. *Clin Exp Allergy.* 2004;34:437-444.
33. Chatila TA. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends Mol Med.* 2004;10:493-499.
34. Wills-Karp M, Chiaramonte M. Interleukin-13 in asthma. *Curr Opin Pulm Med.* 2003;9:21-27.
35. Bosse Y, Thompson C, Audette K, Stankova J, Rola-Pleszczynski M. Interleukin-4 and Interleukin-13 Enhance Human Bronchial Smooth Muscle Cell Proliferation. *Int Arch Allergy Immunol.* 2008;146:138-148.
36. Tedgui A, Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev.* 2006;86:515-581.
37. Dinarello CA. Proinflammatory cytokines. *Chest.* 2000;118:503-508.
38. Andreakos ET, Foxwell BM, Brennan FM, Maini RN, Feldmann M. Cytokines and anti-cytokine biologicals in autoimmunity: present and future. *Cytokine Growth Factor Rev.* 2002;13:299-313.
39. Nasraway SA. The problems and challenges of immunotherapy in sepsis. *Chest.* 2003;123:451S-459S.
40. Lalani I, Bhol K, Ahmed AR. Interleukin-10: biology, role in inflammation and autoimmunity. *Ann Allergy Asthma Immunol.* 1997;79:469-483.
41. Terkeltaub RA. IL-10: An "immunologic scalpel" for atherosclerosis? *Arterioscler Thromb Vasc Biol.* 1999;19:2823-2825.
42. Brennan FM. Interleukin 10 and arthritis. *Rheumatology (Oxford).* 1999;38:293-297.
43. Papadakis KA, Targan SR. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annu Rev Med.* 2000;51:289-298.
44. Lehoux S, Castier Y, Tedgui A. Molecular mechanisms of the vascular responses to haemodynamic forces. *J Intern Med.* 2006;259:381-392.
45. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation.* 2002;105:1135-1143.
46. Kasahara T, Matsushima K. Macrophage signaling, apoptosis, lectins and leukocyte trafficking. *Trends Immunol.* 2001;22:593-594.
47. Aukrust P, Yndestad A, Sandberg WJ, Gullestad L, Damas JK. T cells in coronary artery disease: different effects of different T-cell subsets. *J Am Coll Cardiol.* 2007;50:1459-1461.
48. Mallat Z, Ait-Oufella H, Tedgui A. Regulatory T-cell immunity in atherosclerosis. *Trends Cardiovasc Med.* 2007;17:113-118.
49. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, Flavell RA, Hansson GK, Klatzmann D,

Tedgui A, Mallat Z. Natural regulatory T cells control the development of atherosclerosis in mice. *Nat Med.* 2006;12:178-180.

50. Mor A, Planer D, Luboshits G, Afek A, Metzger S, Chajek-Shaul T, Keren G, George J. Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2007;27:893-900.

51. Wilczynski JR, Radwan M, Kalinka J. The characterization and role of regulatory T cells in immune reactions. *Front Biosci.* 2008;13:2266-2274.

52. Robertson AK, Hansson GK. T cells in atherogenesis: for better or for worse? *Arterioscler Thromb Vasc Biol.* 2006;26:2421-2432.

53. Freigang S, Horkko S, Miller E, Witztum JL, Palinski W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. *Arterioscler Thromb Vasc Biol.* 1998;18:1972-1982.

54. Davenport P, Tipping PG. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. *Am J Pathol.* 2003;163:1117-1125.

55. Daugherty A, Rateri DL. T lymphocytes in atherosclerosis: the yin-yang of Th1 and Th2 influence on lesion formation. *Circ Res.* 2002;90:1039-1040.

56. Harvey EJ, Ramji DP. Interferon-gamma and atherosclerosis: pro- or anti-atherogenic? *Cardiovasc Res.* 2005;67:11-20.

57. Liuzzo G, Kopecky SL, Frye RL, O'Fallon WM, Maseri A, Goronzy JJ, Weyand CM. Perturbation of the T-cell repertoire in patients with unstable angina. *Circulation.* 1999;100:2135-2139.

58. Ranjbaran H, Sokol SI, Gallo A, Eid RE, Iakimov AO, D'Alessio A, Kapoor JR, Akhtar S, Howes CJ, Aslan M, Pfau S, Pober JS, Tellides G. An inflammatory pathway of IFN-gamma production in coronary atherosclerosis. *J Immunol.* 2007;178:592-604.

59. Koga M, Kai H, Yasukawa H, Yamamoto T, Kawai Y, Kato S, Kusaba K, Kai M, Egashira K, Kataoka Y, Imaizumi T. Inhibition of progression and stabilization of plaques by postnatal interferon-gamma function blocking in ApoE-knockout mice. *Circ Res.* 2007;101:348-356.

60. Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest.* 1997;99:2752-2761.

61. Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in apolipoprotein E(-/-) mice through release of interferon-gamma. *Circ Res.* 2002;90:E34-38.

62. Libby P, Warner SJ, Friedman GB. Interleukin 1: a mitogen for human vascular smooth muscle cells that induces the release of growth-inhibitory prostanoids. *J Clin Invest.* 1988;81:487-498.

63. Tellides G, Tereb DA, Kirkiles-Smith NC, Kim RW, Wilson JH, Schechner JS, Lorber MI, Pober JS. Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. *Nature.* 2000;403:207-211.

64. Penn MS, Topol EJ. Tissue factor, the emerging link between inflammation, thrombosis, and vascular remodeling. *Circ Res.* 2001;89:1-2.

65. Falk E, Shah PK, Fuster V. Coronary plaque disruption. *Circulation.* 1995;92:657-671.
66. Farb A, Burke AP, Tang AL, Liang TY, Mannan P, Smialek J, Virmani R. Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death. *Circulation.* 1996;93:1354-1363.
67. Davies MJ. The pathophysiology of acute coronary syndromes. *Heart.* 2000;83:361-366.
68. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation.* 1994;89:36-44.
69. Schoenhagen P, Ziada KM, Kapadia SR, Crowe TD, Nissen SE, Tuzcu EM. Extent and direction of arterial remodeling in stable versus unstable coronary syndromes : an intravascular ultrasound study. *Circulation.* 2000;101:598-603.
70. Takano M, Mizuno K, Okamatsu K, Yokoyama S, Ohba T, Sakai S. Mechanical and structural characteristics of vulnerable plaques: analysis by coronary angioscopy and intravascular ultrasound. *J Am Coll Cardiol.* 2001;38:99-104.
71. von Birgelen C, Klinkhart W, Mintz GS, Papatheodorou A, Herrmann J, Baumgart D, Haude M, Wieneke H, Ge J, Erbel R. Plaque distribution and vascular remodeling of ruptured and nonruptured coronary plaques in the same vessel: an intravascular ultrasound study in vivo. *J Am Coll Cardiol.* 2001;37:1864-1870.
72. Shah PK. Molecular mechanisms of plaque instability. *Curr Opin Lipidol.* 2007;18:492-499.
73. Chen L, Chester MR, Crook R, Kaski JC. Differential progression of complex culprit stenoses in patients with stable and unstable angina pectoris. *J Am Coll Cardiol.* 1996;28:597-603.
74. Buffon A, Biasucci LM, Liuzzo G, D'Onofrio G, Crea F, Maseri A. Widespread coronary inflammation in unstable angina. *N Engl J Med.* 2002;347:5-12.
75. Rioufol G, Finet G, Ginon I, Andre-Fouet X, Rossi R, Vialle E, Desjoyaux E, Convert G, Huret JF, Tabib A. Multiple atherosclerotic plaque rupture in acute coronary syndrome: a three-vessel intravascular ultrasound study. *Circulation.* 2002;106:804-808.
76. Chiu B. Multiple infections in carotid atherosclerotic plaques. *Am Heart J.* 1999;138:S534-536.
77. Fiehn NE, Larsen T, Christiansen N, Holmstrup P, Schroeder TV. Identification of periodontal pathogens in atherosclerotic vessels. *J Periodontol.* 2005;76:731-736.
78. Haynes WG, Stanford C. Periodontal disease and atherosclerosis: from dental to arterial plaque. *Arterioscler Thromb Vasc Biol.* 2003;23:1309-1311.
79. Apfalter P, Barousch W, Nehr M, Willinger B, Rotter M, Hirschl AM. No evidence of involvement of Chlamydia pneumoniae in severe cerebrovascular atherosclerosis by means of quantitative real-time polymerase chain reaction. *Stroke.* 2004;35:2024-2028.
80. Grayston JT. Background and current knowledge of Chlamydia pneumoniae and atherosclerosis. *J Infect Dis.* 2000;181 Suppl 3:S402-410.

81. Saikku P, Leinonen M, Tenkanen L, Linnanmaki E, Ekman MR, Manninen V, Manttari M, Frick MH, Huttunen JK. Chronic Chlamydia pneumoniae infection as a risk factor for coronary heart disease in the Helsinki Heart Study. *Ann Intern Med.* 1992;116:273-278.

82. Kuo CC, Gown AM, Benditt EP, Grayston JT. Detection of Chlamydia pneumoniae in aortic lesions of atherosclerosis by immunocytochemical stain. *Arterioscler Thromb.* 1993;13:1501-1504.

83. Muhlestein JB, Anderson JL, Hammond EH, Zhao L, Trehan S, Schwobe EP, Carlquist JF. Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation.* 1998;97:633-636.

84. Shah PK. Link between infection and atherosclerosis: who are the culprits: viruses, bacteria, both, or neither? *Circulation.* 2001;103:5-6.

85. Xu Q. Role of heat shock proteins in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2002;22:1547-1559.

86. Dybdahl B, Wahba A, Lien E, Flo TH, Waage A, Qureshi N, Sellevold OF, Espevik T, Sundan A. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. *Circulation.* 2002;105:685-690.

87. Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA. Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J Immunol.* 2000;164:13-17.

88. Sasu S, LaVerda D, Qureshi N, Golenbock DT, Beasley D. Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ Res.* 2001;89:244-250.

89. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation.* 2002;105:1158-1161.

90. Xu XH, Shah PK, Faure E, Equils O, Thomas L, Fishbein MC, Luthringer D, Xu XP, Rajavashisth TB, Yano J, Kaul S, Arditi M. Toll-like receptor-4 is expressed by macrophages in murine and human lipid-rich atherosclerotic plaques and upregulated by oxidized LDL. *Circulation.* 2001;104:3103-3108.

91. Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, Schwartz DA. Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med.* 2002;347:185-192.

92. Kiechl S, Wiedermann CJ, Willeit J. Toll-like receptor 4 and atherogenesis. *Ann Med.* 2003;35:164-171.

93. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res.* 2002;91:281-291.

94. Caligiuri G, Paulsson G, Nicoletti A, Maseri A, Hansson GK. Evidence for antigen-driven T-cell response in unstable angina. *Circulation.* 2000;102:1114-1119.

95. Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res.* 1999;85:753-766.

96. Damas JK, Waehre T, Yndestad A, Otterdal K, Hognestad A, Solum NO, Gullestad L, Froland SS, Aukrust P. Interleukin-7-mediated inflammation in unstable angina: possible role of chemokines and platelets. *Circulation.* 2003;107:2670-2676.
97. Wuttge DM, Eriksson P, Sirsjo A, Hansson GK, Stemme S. Expression of interleukin-15 in mouse and human atherosclerotic lesions. *Am J Pathol.* 2001;159:417-423.
98. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol.* 2003;3:745-756.
99. Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol.* 2002;3:221-227.
100. Kasof GM, Lu JJ, Liu D, Speer B, Mongan KN, Gomes BC, Lorenzi MV. Tumor necrosis factor-alpha induces the expression of DR6, a member of the TNF receptor family, through activation of NF-kappaB. *Oncogene.* 2001;20:7965-7975.
101. Ravi R, Bedi GC, Engstrom LW, Zeng Q, Mookerjee B, Gelinas C, Fuchs EJ, Bedi A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-kappaB. *Nat Cell Biol.* 2001;3:409-416.
102. Zheng Y, Ouaaz F, Bruzzo P, Singh V, Gerondakis S, Beg AA. NF-kappa B RelA (p65) is essential for TNF-alpha-induced fas expression but dispensable for both TCR-induced expression and activation-induced cell death. *J Immunol.* 2001;166:4949-4957.
103. Fu YX, Chaplin DD. Development and maturation of secondary lymphoid tissues. *Annu Rev Immunol.* 1999;17:399-433.
104. Darnay BG, Aggarwal BB. Signal transduction by tumour necrosis factor and tumour necrosis factor related ligands and their receptors. *Ann Rheum Dis.* 1999;58 Suppl 1:I2-I13.
105. Panes J, Gomollon F, Taxonera C, Hinojosa J, Clofent J, Nos P. Crohn's disease: a review of current treatment with a focus on biologics. *Drugs.* 2007;67:2511-2537.
106. Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E. SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. *J Biol Chem.* 2001;276:47944-47949.
107. Bolger AP, Anker SD. Tumour necrosis factor in chronic heart failure: a peripheral view on pathogenesis, clinical manifestations and therapeutic implications. *Drugs.* 2000;60:1245-1257.
108. Diwan A, Tran T, Misra A, Mann DL. Inflammatory mediators and the failing heart: a translational approach. *Curr Mol Med.* 2003;3:161-182.
109. Winthrop KL. Risk and prevention of tuberculosis and other serious opportunistic infections associated with the inhibition of tumor necrosis factor. *Nat Clin Pract Rheumatol.* 2006;2:602-610.
110. van Kooten C, Banchereau J. CD40-CD40 ligand. *J Leukoc Biol.* 2000;67:2-17.
111. Ohta H, Wada H, Niwa T, Kirii H, Iwamoto N, Fujii H, Saito K, Sekikawa K, Seishima M. Disruption of tumor necrosis factor-alpha gene diminishes the

development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis.* 2005;180:11-17.

112. Schreyer SA, Peschon JJ, LeBoeuf RC. Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. *J Biol Chem.* 1996;271:26174-26178.

113. Lee WH, Kim SH, Lee Y, Lee BB, Kwon B, Song H, Kwon BS, Park JE. Tumor necrosis factor receptor superfamily 14 is involved in atherogenesis by inducing proinflammatory cytokines and matrix metalloproteinases. *Arterioscler Thromb Vasc Biol.* 2001;21:2004-2010.

114. Grignani G, Maiolo A. Cytokines and hemostasis. *Haematologica.* 2000;85:967-972.

115. Aukrust P, Muller F, Ueland T, Berget T, Aaser E, Brunsvig A, Solum NO, Forfang K, Froland SS, Gullestad L. Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation.* 1999;100:614-620.

116. Schonbeck U, Libby P. CD40 signaling and plaque instability. *Circ Res.* 2001;89:1092-1103.

117. Schonbeck U, Libby P. The CD40/CD154 receptor/ligand dyad. *Cell Mol Life Sci.* 2001;58:4-43.

118. Mach F, Schonbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci U S A.* 1997;94:1931-1936.

119. Schonbeck U, Mach F, Sukhova GK, Atkinson E, Levesque E, Herman M, Graber P, Basset P, Libby P. Expression of stromelysin-3 in atherosclerotic lesions: regulation via CD40-CD40 ligand signaling in vitro and in vivo. *J Exp Med.* 1999;189:843-853.

120. Lutgens E, Gorelik L, Daemen MJ, de Muinck ED, Grewal IS, Koteliansky VE, Flavell RA. Requirement for CD154 in the progression of atherosclerosis. *Nat Med.* 1999;5:1313-1316.

121. Lutgens E, Cleutjens KB, Heeneman S, Koteliansky VE, Burkly LC, Daemen MJ. Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype. *Proc Natl Acad Sci U S A.* 2000;97:7464-7469.

122. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature.* 1998;391:591-594.

123. Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD. CD40L stabilizes arterial thrombi by a beta3 integrin--dependent mechanism. *Nat Med.* 2002;8:247-252.

124. Kinlay S, Schwartz GG, Olsson AG, Rifai N, Sasiela WJ, Szarek M, Ganz P, Libby P. Effect of atorvastatin on risk of recurrent cardiovascular events after an acute coronary syndrome associated with high soluble CD40 ligand in the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) Study. *Circulation.* 2004;110:386-391.

125. Bavendiek U, Zirlik A, LaClair S, MacFarlane L, Libby P, Schonbeck U. Atherogenesis in mice does not require CD40 ligand from bone marrow-derived cells. *Arterioscler Thromb Vasc Biol.* 2005;25:1244-1249.
126. Antman EM, Cohen M, Bernink PJ, McCabe CH, Horacek T, Papuchis G, Mautner B, Corbalan R, Radley D, Braunwald E. The TIMI risk score for unstable angina/non-ST elevation MI: A method for prognostication and therapeutic decision making. *Jama.* 2000;284:835-842.
127. Seidel C, Hjorth-Hansen H, Bendz B, Borset M, Sandset PM, Hansen JB, Sundan A, Waage A. Hepatocyte growth factor in serum after injection of unfractionated and low molecular weight heparin in healthy individuals. *Br J Haematol.* 1999;105:641-647.
128. Cannon JG, Nerad JL, Poutsiaka DD, Dinarello CA. Measuring circulating cytokines. *J Appl Physiol.* 1993;75:1897-1902.
129. Riches P, Gooding R, Millar BC, Rowbottom AW. Influence of collection and separation of blood samples on plasma IL-1, IL-6 and TNF-alpha concentrations. *J Immunol Methods.* 1992;153:125-131.
130. van Wanrooij EJ, van Puijvelde GH, de Vos P, Yagita H, van Berkel TJ, Kuiper J. Interruption of the Tnfrsf4/Tnfsf4 (OX40/OX40L) pathway attenuates atherogenesis in low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol.* 2007;27:204-210.
131. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature.* 1997;390:175-179.
132. Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature.* 1999;402:304-309.
133. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature.* 1999;397:315-323.
134. Lum L, Reid MS, Blobel CP. Intracellular maturation of the mouse metalloprotease disintegrin MDC15. *J Biol Chem.* 1998;273:26236-26247.
135. Theill LE, Boyle WJ, Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol.* 2002;20:795-823.
136. Weitzmann MN, Cenci S, Rifas L, Brown C, Pacifici R. Interleukin-7 stimulates osteoclast formation by up-regulating the T-cell production of soluble osteoclastogenic cytokines. *Blood.* 2000;96:1873-1878.
137. Teng YT, Nguyen H, Gao X, Kong YY, Gorczynski RM, Singh B, Ellen RP, Penninger JM. Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. *J Clin Invest.* 2000;106:R59-67.

138. Hofbauer LC, Heufelder AE. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J Mol Med.* 2001;79:243-253.
139. Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. *Jama.* 2004;292:490-495.
140. Sattler AM, Schoppet M, Schaefer JR, Hofbauer LC. Novel aspects on RANK ligand and osteoprotegerin in osteoporosis and vascular disease. *Calcif Tissue Int.* 2004;74:103-106.
141. Walsh MC, Choi Y. Biology of the TRANCE axis. *Cytokine Growth Factor Rev.* 2003;14:251-263.
142. Kanamaru F, Iwai H, Ikeda T, Nakajima A, Ishikawa I, Azuma M. Expression of membrane-bound and soluble receptor activator of NF-kappaB ligand (RANKL) in human T cells. *Immunol Lett.* 2004;94:239-246.
143. Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res.* 2004;95:1046-1057.
144. Jono S, Ikari Y, Shioi A, Mori K, Miki T, Hara K, Nishizawa Y. Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. *Circulation.* 2002;106:1192-1194.
145. Avignon A, Sultan A, Piot C, Elaerts S, Cristol JP, Dupuy AM. Osteoprotegerin is associated with silent coronary artery disease in high-risk but asymptomatic type 2 diabetic patients. *Diabetes Care.* 2005;28:2176-2180.
146. Schoppet M, Sattler AM, Schaefer JR, Herzum M, Maisch B, Hofbauer LC. Increased osteoprotegerin serum levels in men with coronary artery disease. *J Clin Endocrinol Metab.* 2003;88:1024-1028.
147. Ueland T, Jemtland R, Godang K, Kjekshus J, Hognestad A, Omland T, Squire IB, Gullestad L, Bollerslev J, Dickstein K, Aukrust P. Prognostic value of osteoprotegerin in heart failure after acute myocardial infarction. *J Am Coll Cardiol.* 2004;44:1970-1976.
148. Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J Exp Med.* 1992;175:323-329.
149. Aukrust P, Lien E, Kristoffersen AK, Muller F, Haug CJ, Espevik T, Froland SS. Persistent activation of the tumor necrosis factor system in a subgroup of patients with common variable immunodeficiency--possible immunologic and clinical consequences. *Blood.* 1996;87:674-681.
150. Carter A, Haddad N, Draxler I, Tatarsky I. Effects of soluble interleukin-1 receptor and tumor-necrosis factor receptor, respectively, on the IL-1- and TNF-alpha-induced DNA synthesis of acute myeloblastic leukemia blasts in vitro. *Eur J Haematol.* 1994;53:38-45.
151. Howard OM, Clouse KA, Smith C, Goodwin RG, Farrar WL. Soluble tumor necrosis factor receptor: inhibition of human immunodeficiency virus activation. *Proc Natl Acad Sci U S A.* 1993;90:2335-2339.
152. Bennett BJ, Scatena M, Kirk EA, Rattazzi M, Varon RM, Averill M, Schwartz SM, Giachelli CM, Rosenfeld ME. Osteoprotegerin inactivation accelerates advanced atherosclerotic lesion progression and calcification in older ApoE-/- mice. *Arterioscler Thromb Vasc Biol.* 2006;26:2117-2124.

153. Golledge J, McCann M, Mangan S, Lam A, Karan M. Osteoprotegerin and osteopontin are expressed at high concentrations within symptomatic carotid atherosclerosis. *Stroke.* 2004;35:1636-1641.
154. Breuil V, Schmid-Antomarchi H, Schmid-Alliana A, Rezzonico R, Euller-Ziegler L, Rossi B. The receptor activator of nuclear factor (NF)-kappaB ligand (RANKL) is a new chemotactic factor for human monocytes. *Faseb J.* 2003;17:1751-1753.
155. Kim HH, Shin HS, Kwak HJ, Ahn KY, Kim JH, Lee HJ, Lee MS, Lee ZH, Koh GY. RANKL regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. *Faseb J.* 2003;17:2163-2165.
156. Morony S, Tintut Y, Zhang Z, Cattley RC, Van G, Dwyer D, Stolina M, Kostenuik PJ, Demer LL. Osteoprotegerin inhibits vascular calcification without affecting atherosclerosis in ldlr(-/-) mice. *Circulation.* 2008;117:411-420.
157. Boehlen F, Clemetson KJ. Platelet chemokines and their receptors: what is their relevance to platelet storage and transfusion practice? *Transfus Med.* 2001;11:403-417.
158. Brandt E, Ludwig A, Petersen F, Flad HD. Platelet-derived CXC chemokines: old players in new games. *Immunol Rev.* 2000;177:204-216.
159. Power CA, Clemetson JM, Clemetson KJ, Wells TN. Chemokine and chemokine receptor mRNA expression in human platelets. *Cytokine.* 1995;7:479-482.
160. Smith C, Damas JK, Otterdal K, Oie E, Sandberg WJ, Yndestad A, Waehre T, Scholz H, Endresen K, Olofsson PS, Halvorsen B, Gullestad L, Froland SS, Hansson GK, Aukrust P. Increased levels of neutrophil-activating peptide-2 in acute coronary syndromes: possible role of platelet-mediated vascular inflammation. *J Am Coll Cardiol.* 2006;48:1591-1599.
161. Gawaz M, Brand K, Dickfeld T, Pogatsa-Murray G, Page S, Bogner C, Koch W, Schomig A, Neumann F. Platelets induce alterations of chemotactic and adhesive properties of endothelial cells mediated through an interleukin-1-dependent mechanism. Implications for atherogenesis. *Atherosclerosis.* 2000;148:75-85.
162. Loppnow H, Bil R, Hirt S, Schonbeck U, Herzberg M, Werdan K, Rietschel ET, Brandt E, Flad HD. Platelet-derived interleukin-1 induces cytokine production, but not proliferation of human vascular smooth muscle cells. *Blood.* 1998;91:134-141.
163. Clemetson KJ, Clemetson JM, Proudfoot AE, Power CA, Baggiolini M, Wells TN. Functional expression of CCR1, CCR3, CCR4, and CXCR4 chemokine receptors on human platelets. *Blood.* 2000;96:4046-4054.
164. von Hundelshausen P, Weber KS, Huo Y, Proudfoot AE, Nelson PJ, Ley K, Weber C. RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. *Circulation.* 2001;103:1772-1777.
165. Aukrust P, Waehre T, Damas JK, Gullestad L, Solum NO. Inflammatory role of platelets in acute coronary syndromes. *Heart.* 2001;86:605-606.
166. Damas JK, Jensenius M, Ueland T, Otterdal K, Yndestad A, Froland SS, Rolain JM, Myrvang B, Raoult D, Aukrust P. Increased levels of soluble CD40L in African tick bite fever: possible involvement of TLRs in the pathogenic interaction between Rickettsia africae, endothelial cells, and platelets. *J Immunol.* 2006;177:2699-2706.

167. Waehre T, Damas JK, Yndestad A, Tasken K, Pedersen TM, Smith C, Halvorsen B, Froland SS, Solum NO, Aukrust P. Effect of activated platelets on expression of cytokines in peripheral blood mononuclear cells - potential role of prostaglandin E2. *Thromb Haemost.* 2004;92:1358-1367.
168. Ahmad R, Menezes J, Knafo L, Ahmad A. Activated human platelets express Fas-L and induce apoptosis in Fas-positive tumor cells. *J Leukoc Biol.* 2001;69:123-128.
169. Crist SA, Elzey BD, Ludwig AT, Griffith TS, Staack JB, Lentz SR, Ratliff TL. Expression of TNF-related apoptosis-inducing ligand (TRAIL) in megakaryocytes and platelets. *Exp Hematol.* 2004;32:1073-1081.
170. Otterdal K, Smith C, Oie E, Pedersen TM, Yndestad A, Stang E, Endresen K, Solum NO, Aukrust P, Damas JK. Platelet-derived LIGHT induces inflammatory responses in endothelial cells and monocytes. *Blood.* 2006;108:928-935.
171. Aukrust P, Damas JK, Solum NO. Soluble CD40 ligand and platelets: self-perpetuating pathogenic loop in thrombosis and inflammation? *J Am Coll Cardiol.* 2004;43:2326-2328.
172. Zhang Y, Cao HJ, Graf B, Meekins H, Smith TJ, Phipps RP. CD40 engagement up-regulates cyclooxygenase-2 expression and prostaglandin E2 production in human lung fibroblasts. *J Immunol.* 1998;160:1053-1057.
173. Damas JK, Otterdal K, Yndestad A, Aass H, Solum NO, Froland SS, Simonsen S, Aukrust P, Andreassen AK. Soluble CD40 ligand in pulmonary arterial hypertension: possible pathogenic role of the interaction between platelets and endothelial cells. *Circulation.* 2004;110:999-1005.
174. Prasad KS, Andre P, He M, Bao M, Manganello J, Phillips DR. Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. *Proc Natl Acad Sci U S A.* 2003;100:12367-12371.
175. Furman MI, Krueger LA, Linden MD, Barnard MR, Frelinger AL, 3rd, Michelson AD. Release of soluble CD40L from platelets is regulated by glycoprotein IIb/IIIa and actin polymerization. *J Am Coll Cardiol.* 2004;43:2319-2325.
176. Nannizzi-Alaimo L, Alves VL, Phillips DR. Inhibitory effects of glycoprotein IIb/IIIa antagonists and aspirin on the release of soluble CD40 ligand during platelet stimulation. *Circulation.* 2003;107:1123-1128.
177. Otterdal K, Pedersen TM, Solum NO. Release of soluble CD40 ligand after platelet activation: studies on the solubilization phase. *Thromb Res.* 2004;114:167-177.
178. Lopez-Fraga M, Fernandez R, Albar JP, Hahne M. Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. *EMBO Rep.* 2001;2:945-951.
179. Macaulay IC, Carr P, Gusnanto A, Ouwehand WH, Fitzgerald D, Watkins NA. Platelet genomics and proteomics in human health and disease. *J Clin Invest.* 2005;115:3370-3377.
180. Ingold K, Zumsteg A, Tardivel A, Huard B, Steiner QG, Cachero TG, Qiang F, Gorelik L, Kalled SL, Acha-Orbea H, Rennert PD, Tschopp J, Schneider P. Identification of proteoglycans as the APRIL-specific binding partners. *J Exp Med.* 2005;201:1375-1383.

181. Schneider P. The role of APRIL and BAFF in lymphocyte activation. *Curr Opin Immunol.* 2005;17:282-289.
182. Mackay F, Leung H. The role of the BAFF/APRIL system on T cell function. *Semin Immunol.* 2006;18:284-289.
183. Dillon SR, Gross JA, Ansell SM, Novak AJ. An APRIL to remember: novel TNF ligands as therapeutic targets. *Nat Rev Drug Discov.* 2006;5:235-246.
184. Koyama T, Tsukamoto H, Miyagi Y, Himeji D, Otsuka J, Miyagawa H, Harada M, Horiuchi T. Raised serum APRIL levels in patients with systemic lupus erythematosus. *Ann Rheum Dis.* 2005;64:1065-1067.
185. Jonsson MV, Szodoray P, Jellestad S, Jonsson R, Skarstein K. Association between circulating levels of the novel TNF family members APRIL and BAFF and lymphoid organization in primary Sjogren's syndrome. *J Clin Immunol.* 2005;25:189-201.
186. Mariette X, Roux S, Zhang J, Bengoufa D, Lavie F, Zhou T, Kimberly R. The level of BLyS (BAFF) correlates with the titre of autoantibodies in human Sjogren's syndrome. *Ann Rheum Dis.* 2003;62:168-171.
187. Tan SM, Xu D, Roschke V, Perry JW, Arkfeld DG, Ehresmann GR, Migone TS, Hilbert DM, Stohl W. Local production of B lymphocyte stimulator protein and APRIL in arthritic joints of patients with inflammatory arthritis. *Arthritis Rheum.* 2003;48:982-992.
188. Zhou X, Hansson GK. Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice. *Scand J Immunol.* 1999;50:25-30.
189. Hansson GK, Robertson AK, Soderberg-Naucler C. Inflammation and atherosclerosis. *Annu Rev Pathol.* 2006;1:297-329.
190. Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Invest.* 2002;109:745-753.
191. Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, Shaw PX, Palinski W, Witztum JL, Silverman GJ. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med.* 2003;9:736-743.
192. Belnoue E, Pihlgren M, McGaha TL, Tougne C, Rochat AF, Bossen C, Schneider P, Huard B, Lambert PH, Siegrist CA. APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells. *Blood.* 2008;111:2755-2764.
193. Mardla V, Kobzar G, Samel N. Potentiation of antiaggregating effect of prostaglandins by alpha-tocopherol and quercetin. *Platelets.* 2004;15:319-324.
194. Ramanujam M, Wang X, Huang W, Schiffer L, Grimaldi C, Akkerman A, Diamond B, Madaio MP, Davidson A. Mechanism of action of transmembrane activator and calcium modulator ligand interactor-Ig in murine systemic lupus erythematosus. *J Immunol.* 2004;173:3524-3534.
195. Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, Ruben S, Murphy M, Eisenberg RJ, Cohen GH, Spear PG, Ware CF. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity.* 1998;8:21-30.

196. Zhai Y, Guo R, Hsu TL, Yu GL, Ni J, Kwon BS, Jiang GW, Lu J, Tan J, Ugustus M, Carter K, Rojas L, Zhu F, Lincoln C, Endress G, Xing L, Wang S, Oh KO, Gentz R, Ruben S, Lippman ME, Hsieh SL, Yang D. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. *J Clin Invest.* 1998;102:1142-1151.

197. Yu KY, Kwon B, Ni J, Zhai Y, Ebner R, Kwon BS. A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. *J Biol Chem.* 1999;274:13733-13736.

198. Chen MC, Hwang MJ, Chou YC, Chen WH, Cheng G, Nakano H, Luh TY, Mai SC, Hsieh SL. The role of apoptosis signal-regulating kinase 1 in lymphotoxin-beta receptor-mediated cell death. *J Biol Chem.* 2003;278:16073-16081.

199. Morel Y, Schiano de Colella JM, Harrop J, Deen KC, Holmes SD, Wattam TA, Khandekar SS, Truneh A, Sweet RW, Gastaut JA, Olive D, Costello RT. Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. *J Immunol.* 2000;165:4397-4404.

200. Rooney IA, Butrovich KD, Glass AA, Borboroglu S, Benedict CA, Whitbeck JC, Cohen GH, Eisenberg RJ, Ware CF. The lymphotoxin-beta receptor is necessary and sufficient for LIGHT-mediated apoptosis of tumor cells. *J Biol Chem.* 2000;275:14307-14315.

201. Scheu S, Alferink J, Potzel T, Barchet W, Kalinke U, Pfeffer K. Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin beta in mesenteric lymph node genesis. *J Exp Med.* 2002;195:1613-1624.

202. Tamada K, Shimozaki K, Chapoval AI, Zhai Y, Su J, Chen SF, Hsieh SL, Nagata S, Ni J, Chen L. LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. *J Immunol.* 2000;164:4105-4110.

203. Kwon BS, Tan KB, Ni J, Oh KO, Lee ZH, Kim KK, Kim YJ, Wang S, Gentz R, Yu GL, Harrop J, Lyn SD, Silverman C, Porter TG, Truneh A, Young PR. A newly identified member of the tumor necrosis factor receptor superfamily with a wide tissue distribution and involvement in lymphocyte activation. *J Biol Chem.* 1997;272:14272-14276.

204. Granger SW, Rickert S. LIGHT-HVEM signaling and the regulation of T cell-mediated immunity. *Cytokine Growth Factor Rev.* 2003;14:289-296.

205. Shaikh RB, Santee S, Granger SW, Butrovich K, Cheung T, Kronenberg M, Cheroutre H, Ware CF. Constitutive expression of LIGHT on T cells leads to lymphocyte activation, inflammation, and tissue destruction. *J Immunol.* 2001;167:6330-6337.

206. Kosuge H, Suzuki J, Kakuta T, Haraguchi G, Koga N, Futamatsu H, Gotoh R, Inobe M, Isobe M, Uede T. Attenuation of graft arterial disease by manipulation of the LIGHT pathway. *Arterioscler Thromb Vasc Biol.* 2004;24:1409-1415.

207. Wei CY, Chou YH, Ho FM, Hsieh SL, Lin WW. Signaling pathways of LIGHT induced macrophage migration and vascular smooth muscle cell proliferation. *J Cell Physiol.* 2006;209:735-743.

208. Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol.* 2008;9:176-185.
209. Chang YH, Hsieh SL, Chao Y, Chou YC, Lin WW. Proinflammatory effects of LIGHT through HVEM and LTbetaR interactions in cultured human umbilical vein endothelial cells. *J Biomed Sci.* 2005;12:363-375.
210. Ferrell WR, Lockhart JC, Kelso EB, Dunning L, Plevin R, Meek SE, Smith AJ, Hunter GD, McLean JS, McGarry F, Ramage R, Jiang L, Kanke T, Kawagoe J. Essential role for proteinase-activated receptor-2 in arthritis. *J Clin Invest.* 2003;111:35-41.
211. Kelso EB, Lockhart JC, Hembrough T, Dunning L, Plevin R, Hollenberg MD, Sommerhoff CP, McLean JS, Ferrell WR. Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. *J Pharmacol Exp Ther.* 2006;316:1017-1024.
212. Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS, Luger TA, Schmelz M. Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. *J Neurosci.* 2003;23:6176-6180.
213. Namazi MR. Possible molecular mechanisms to account for the involvement of tryptase in the pathogenesis of psoriasis. *Autoimmunity.* 2005;38:449-452.
214. Cocks TM, Fong B, Chow JM, Anderson GP, Frauman AG, Goldie RG, Henry PJ, Carr MJ, Hamilton JR, Moffatt JD. A protective role for protease-activated receptors in the airways. *Nature.* 1999;398:156-160.
215. Kawabata A, Kawao N. Physiology and pathophysiology of proteinase-activated receptors (PARs): PARs in the respiratory system: cellular signaling and physiological/pathological roles. *J Pharmacol Sci.* 2005;97:20-24.
216. Morello S, Vellecco V, Roviezzo F, Maffia P, Cuzzocrea S, Cirino G, Cicala C. A protective role for proteinase activated receptor 2 in airways of lipopolysaccharide-treated rats. *Biochem Pharmacol.* 2005;71:223-230.
217. Fiorucci S, Mencarelli A, Palazzetti B, Distrutti E, Vergnolle N, Hollenberg MD, Wallace JL, Morelli A, Cirino G. Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. *Proc Natl Acad Sci U S A.* 2001;98:13936-13941.
218. Kawabata A, Kinoshita M, Nishikawa H, Kuroda R, Nishida M, Araki H, Arizono N, Oda Y, Kakehi K. The protease-activated receptor-2 agonist induces gastric mucus secretion and mucosal cytoprotection. *J Clin Invest.* 2001;107:1443-1450.
219. Schneider K, Potter KG, Ware CF. Lymphotoxin and LIGHT signaling pathways and target genes. *Immunol Rev.* 2004;202:49-66.
220. Tamada K, Shimozaki K, Chapoval AI, Zhu G, Sica G, Flies D, Boone T, Hsu H, Fu YX, Nagata S, Ni J, Chen L. Modulation of T-cell-mediated immunity in tumor and graft-versus-host disease models through the LIGHT co-stimulatory pathway. *Nat Med.* 2000;6:283-289.
221. Kim YS, Nedospasov SA, Liu ZG. TRAF2 plays a key, nonredundant role in LIGHT-lymphotoxin beta receptor signaling. *Mol Cell Biol.* 2005;25:2130-2137.
222. Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, Takashima Y, Kawabe Y, Cynshi O, Wada Y, Honda M, Kurihara H, Aburatani H, Doi T, Matsumoto A, Azuma S, Noda T,

Toyoda Y, Itakura H, Yazaki Y, Kodama T, et al. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature.* 1997;386:292-296.

223. Babaev VR, Gleaves LA, Carter KJ, Suzuki H, Kodama T, Fazio S, Linton MF. Reduced atherosclerotic lesions in mice deficient for total or macrophage-specific expression of scavenger receptor-A. *Arterioscler Thromb Vasc Biol.* 2000;20:2593-2599.

224. Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF, Freeman MW. Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem.* 2002;277:49982-49988.

225. Fukuhara-Takaki K, Sakai M, Sakamoto Y, Takeya M, Horiuchi S. Expression of class A scavenger receptor is enhanced by high glucose in vitro and under diabetic conditions in vivo: one mechanism for an increased rate of atherosclerosis in diabetes. *J Biol Chem.* 2005;280:3355-3364.

226. Linton MF, Fazio S. Class A scavenger receptors, macrophages, and atherosclerosis. *Curr Opin Lipidol.* 2001;12:489-495.

227. Lo JC, Wang Y, Tumanov AV, Bamji M, Yao Z, Reardon CA, Getz GS, Fu YX. Lymphotoxin beta receptor-dependent control of lipid homeostasis. *Science.* 2007;316:285-288.

228. Moreno PR, Bernardi VH, Lopez-Cuellar J, Murcia AM, Palacios IF, Gold HK, Mehran R, Sharma SK, Nemerson Y, Fuster V, Fallon JT. Macrophages, smooth muscle cells, and tissue factor in unstable angina. Implications for cell-mediated thrombogenicity in acute coronary syndromes. *Circulation.* 1996;94:3090-3097.

229. Schwager I, Jungi TW. Effect of human recombinant cytokines on the induction of macrophage procoagulant activity. *Blood.* 1994;83:152-160.